Evaluation of soil bacteria as bioinoculants for the control of field pea root rot caused by *Aphanomyces euteiches*

A Thesis Submitted to the

College of Graduate and Postdoctoral Studies

in Partial Fulfilment of the Requirements

for the Degree of Master of Science

in the Department of Soil Science

University of Saskatchewan

Saskatoon

By

Ashebir Tsedeke Godebo

PERMISSION TO USE

In presenting this thesis in partial fulfilment of the requirement for a post graduate degree from the University of Saskatchewan, I agree that the library of this University may take it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purpose may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work is done. It is understood that any copying or publication or use of this thesis part or its parts for financial gain shall not be allowed without my written permission. It is also understood that due consideration shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Request for permission to copy or to make other use of any material in this thesis in whole or part should be addressed to:

Head of the Department of Soil Science

51 Campus drive

University of Saskatchewan

Saskatoon, Saskatchewan S7N 5A8 Canada

OR

Dean College of Graduate and Postdoctoral Studies

University of Saskatchewan

116 Thorvaldson Building, 110 Science Place

Saskatoon, Saskatchewan S7N 5C9 Canada

DISCLAIMER

Reference in this thesis to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not constitute or imply its endorsement, recommendation, or favoring by the University of Saskatchewan. The views and opinions of the author expressed herein do not state or reflect those of the University of Saskatchewan and shall not be used for advertising or product endorsement purposes.

ABSTRACT

Aphanomyces euteiches is an oomycete pathogen that is becoming a serious problem for field pea (*Pisum sativum L*) production in western Canada. The pathogen causes severe rot in the root, cortex, and epicotyl of field pea resulting in stunting, yellow and wilting leaves, or plant death. Aphanomyces root rot develops because of zoosporic or myceliogenic infection when oospores germinate to form germ sporangia and germ tubes, respectively. Until the recent introduction of the fungicide INTEGO™ Solo (ethaboxam) and Vibrance® Maxx RFC, there was no fungicide available in Canada that effectively suppresses or controls aphanomyces root rot in field pea.

Additional control measures are needed, and one addition to chemical control is the development of bacterial inoculants that interrupt the pathogen's lifecycle and ultimately control or reduces disease expression in the host plant. This study comprised laboratory studies aimed at isolating and identifying antagonistic bacteria against *A. euteiches* mycelia and zoospore growth stages, and growth chamber trials that examined efficacy of antagonistic bacteria as biocontrol agents against aphanomyces root rot in field pea.

Soils were collected from 43 commercial field pea fields across Saskatchewan. Initial screening of antagonistic bacteria was completed by assessing mycelia growth inhibition of *A. euteiches in vitro*. Growth inhibition of each antagonistic bacterium was further evaluated using a dual plate assay technique where single colonies of the antagonistic bacterial isolates were inoculated at two opposite edges on a PDA plate and a plug of *A. euteiches* mycelia was placed in the center of the plate. Additionally, a preliminary screening assay utilizing a dual plate technique was employed to assess 170 bacterial isolates for biocontrol activity against *A. euteiches*. These

170 bacterial isolates were from a previously existing bacteria culture collection of roots associated rhizobacteria from a variety of field crops. The antagonistic bacterial isolates were also assayed for *in vitro* zoospore germination inhibition.

A total of 184 antagonistic bacteria, of which 22 were from a previously existing bacteria culture collection, that inhibited the mycelia stage of *A. euteiches* were identified using the initial screening assay. Of these, 47 inhibited zoospore germination by 75% or more compared to a control assay plate. The mean mycelial growth inhibition potential of the isolates ranged from 1 mm to 12 mm whereas the mean zoospore germination inhibition potential of the isolates ranged from 0 to 100 %. Based on 16S rDNA gene sequencing, the antagonistic bacterial isolates were placed into 18 different genera with *Bacillus*, *Pseudomonas*, *Paenibacillus, Lysobacter* and *Streptomyces* being the top five containing 45, 32, 29,17 and 12 antagonistic bacterial isolates, respectively.

Bacterial isolates that inhibited mycelia growth and zoospore germination by 75% or more were selected for further evaluation in growth chamber trials. In the first set of experiments (Trial 1) pea plants were grown in vermiculite and inoculated with antagonistic bacterial suspensions and *A. euteiches* zoospores. Four weeks after planting, the pea plants were harvested, and roots assessed for level of disease development. Isolates which significantly suppressed aphanomyces root rot in vermiculite were further evaluated as soil inoculants in pot experiments using non-sterile field soil (Trial 2).

Screening of 47 antagonistic bacteria as bioinoculants in growth chamber Trial 1 identified 29 that significantly ($\alpha = 0.05$) suppressed or reduced aphanomyces root rot in field pea. Of these, 20 isolates were selected and screened as soil inoculants in a second set of experiments (Trial 2)

and three isolates produced the highest biocontrol activity and significantly ($\alpha = 0.05$) suppressed or reduced aphanomyces root rot in field pea.

From the findings of this research, it can be concluded that the *A. euteiches* lifecycle can be interrupted using rhizosphere bacteria and hence these bacterial isolates may be used as biocontrol agents to suppress or reduce aphanomyces root rot in field pea. Variations of inhibition potential among isolates suggests that the mechanisms by which biocontrol is achieved such as the production and secretion of inhibitory compounds and/or the mode of action exerted by the inhibitory metabolites likely varies among isolates. The results of this research indicate the potential promise for the development of microbial biocontrol agents. Further studies aimed at assessing the efficacy of the promising isolates under field conditions in Saskatchewan and other manipulative studies that would maximize biocontrol potential and their effective utilization are a necessary next step.

ACKNOWLEDGMENTS

I am truly thankful to my supervisors Drs. Fran Walley and Jim Germida for their continuous help and support throughout my program at the University of Saskatchewan. Without their guidance and help, I wouldn't have been able to excel in my studies and research. I would also like to thank and greatly appreciate the time and help given by other committee members, Drs. Sabine Banniza and Jeff Schoenau.

I am grateful to Dr. Bobbi Helgeson and people in Soil Microbiology lab. A very special thanks go to Jorge Cordero, Eduardo Kovalski, Akeem Shorunke, Claire Kohout, Zayda Morales, Bethany Templeton, Panchali Katalunda and the summer students Ian Hnatowich and Kyler Kanegawa. This also extends to Nimllash Sivachandrakumar and Laura Cox from Dr. Sabine's lab who provided technical help. I would also like to thank Marc St. Arnaud and Kim Heidinger from the Department of Soil Science for the administrative assistance.

Finally, I would like to thank the Saskatchewan Pulse Growers for supporting this project financially.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF EQUATIONS

1. GENERAL INTRODUCTION

Field pea (*Pisum sativum L*) belongs to the family of cool season grain legume crops known as pulses, which includes lentil (*Lens culinaris* Medik.), faba bean (*Vicia faba* L.), common bean (*Phaseolus vulgaris* L.) and chickpea (*Cicer arietinum* L.) (Felix et al., 2017). Field pea is appreciated for its nutritive value and is commonly consumed throughout the world and is popular in human vegetarian diets. Pea also is excellent livestock feed as it contains up to 87% total digestible nutrients and high levels of carbohydrates (Gregory et al., 2016).

Canada is the major field pea producing country in the world (Government of Saskatchewan, 2012). Field pea can be grown in no-till or conventional-till cropping systems on soil types ranging from light sandy to heavy clay, although poorly drained and saline soils are not ideal conditions for growth (Gregory et al., 2016). Field pea has a hypogeal emergence and the cotyledon remains below the soil surface and emergence normally occurs within 10 to 14 d. The root system is relatively shallow and small. Although 75% of the root biomass is within 60 cm of the soil surface, the roots can grow up to a depth of 90 to 120 cm (McKay et al., 2003). In western Canada and the Northern USA, the days to maturity can differ depending on the variety. Determinate varieties typically mature between 80 to 90 d whereas indeterminate types mature between 90 to 100 d (Njoka, 2008; Spies, 2008).

Field pea is one of the most effective nitrogen-fixing legumes and under favorable conditions can fix up to 80 % of its total nitrogen (N) requirement from the atmosphere by forming a symbiotic relationship with *Rhizobium* bacteria in the soil (Gregory et al., 2016). For this symbiotic relationship to occur, inoculation of the root with suitable *Rhizobium* strains is required (McKay

et al., 2003; Felix et al., 2017). Nodules develop within two to four weeks of emergence and effective nodules are characterised by pink to red coloration in the interior (McKay et al., 2003).

In Canada, pea cultivation was first introduced during the late 1800s in the eastern part of the country and starting in the 1990s, field pea cultivation significantly increased in North America (Canada and the United States) (Slinkard et al., 1994). Field pea is a well-known cash crop in western Canada and peas are exported to various international markets. Although Canada is the world's largest field pea producer, in recent years there has been a decline in production. For example, field pea production in Saskatchewan reached a high of 1.1 million ha in 2005 (Spies, 2008) but more recently production has decreased to 867,000 ha in 2017 (Canadian Grain Commission, 2017).

Field pea diseases caused by either fungi, bacteria, viruses or nematodes are usually the cause for low productivity and quality (Spies, 2008). Moreover, some diseases are highly destructive and management options are limited. One such disease is aphanomyces root rot caused by the soil-borne pathogen known as *Aphanomyces euteiches* Drechs. Taxonomically, *A. euteiches* is classified under the kingdom Chromista, class Oomycota (as reviewed in Wu et al., 2018). Considering morphological and physiological traits, *A. euteiches* resembles fungi but phylogenetically is related to diatoms, chromophyte algae and other heterokonts (Gaulin et al., 2007). Although field pea is the most susceptible crop, the *A. euteiches* host range includes dry bean, lentil, faba bean and cicer milkvetch (Vandemark and Porter, 2010).

Aphanomyces root rot is a major problem in different pea growing regions of the world. This pathogen is characterised by the formation of thick-walled oospores that can live for more than 10 years and can cause root rot disease at all field pea growth stages (as reviewed in Wu et al., 2018;

Hughes and Grau, 2013). According to Banniza et al. (2013), although aphanomyces root rot was known to exist in Alberta and Manitoba, it was first reported in Saskatchewan in 2012. Since then, because of its damaging nature and occurrence in various pea fields, it has become an emerging concern in western Canada. *Aphanomyces euteiches* has two infective stages during which zoospores and mycelia are produced (Wakelin et al., 2002). Following infection, the pea root produces a honey-colored watery lesion that can progress to severe root rot and at later stages the plant root turns dark brown due to disease progress and secondary infection (Hughes and Grau, 2013). In some extreme cases dwarfing and death of the entire plant can occur. Reports have indicated that in infested soil loss of productivity due to this pathogen can reach up to 100 % (Gaulin et al., 2007).

Aphanomyces root rot not only has limited options for management but also none of the available approaches provide effective and complete protection. The available options for management are crop rotation, disease avoidance (Wakelin et al., 2002; Hughes and Grau, 2013; Wu et al., 2018), and the application of fungicides INTEGO™ Solo (ethaboxam) or Vibrance® Maxx RFC (Sedaxane, Metalaxyl-M and S-isomer and Fludioxonil) which are registered for use against early season aphanomyces root rot in field pea (Guide for Crop Protection, 2018). Moreover, a resistant pea cultivar is absent (as reviewed in Wu et al., 2018). Currently, crop rotation as a control method is under question because oospores can persist in the soil for more than 10 years and have the capacity to build up quickly when a susceptible crop is planted. The lack of an effective control method has resulted in unpreventable crop losses and an economic disadvantage to growers. As a result, there is a need to develop effective control methods that can avoid or reduce aphanomyces root rot development. Otherwise, farmers are reluctant to select field pea as a cropping choice and this could have multifaceted consequences which encompass

sustainability and productivity in addition to the commercial value of the pea crop throughout most western Canadian provinces. One of the approaches to develop an effective management strategy is to consider the use of soil bacteria as a biocontrol agent.

When considering the development of a biological control agent against aphanomyces root rot in field pea, it may be useful to target infective stages within the lifecycle of the pathogen. Interruption of these developmental phases may ultimately provide protection to the plant. Another important consideration is the selection of the type of organism. The types of microorganisms must be the ones which are naturally present in and presumably adapted to the soil immediately surrounding the pea root (i.e., rhizosphere soil). Because rhizosphere bacteria compete effectively for root exudates and are adapted to living in close association with the host plant roots, they should be screened for biological control activity against soil-borne pathogens like *A. euteiches*. Such consideration will increase the chance of finding isolates adapted to the pea rhizosphere environment where the *A. euteiches* lifecycle occurs with the goal of artificially achieving a new balance within the field pea rhizosphere microbial community.

1.1 GENERAL OBJECTIVE

Several researchers have indicated that *A. euteiches* can be controlled using microbial antagonists (Chan, 1985; Bowers and Parke, 1993; Wakelin et al., 2002). Given the growing incidence of aphanomyces root rot and the focus on sustainable pea production in Canada, research into the control of *A. euteiches* in Canada is critically important. The overall aim of the project is to investigate the potential for biological control of aphanomyces root rot. The following specific objectives were developed which outline the structure of the project and this thesis.

1.1.1 Specific objectives

The specific objectives of this project were:

1. To isolate bacteria capable of inhibiting *A. euteiches* mycelial growth from pea field soils;
2. To identify bacteria capable of inhibiting *A. euteiches* zoospore germination;
3. To determine the identity of the antagonistic bacteria using molecular techniques;
4. To evaluate the efficacy of the antagonistic bacteria in suppressing aphanomyces root rot of field pea in a pot experiment using vermiculite;
5. To evaluate the efficacy of the antagonistic bacteria in suppressing aphanomyces root rot of field pea in a pot experiment using non-sterile field soil.

1.2 ORGANIZATION OF THE THESIS

The thesis begins with a review of current literatures. The research is then presented in two chapters following manuscript-style thesis preparation, with both research chapters addressing more than one of the above hypotheses. Each chapter begins with a brief summary of the research (i.e., abstract), a brief introduction that includes a review of the relevant literature, a detailed materials and methods section that contains enough detail that other workers could repeat the work, the data collection and analysis, a summary of the results, and a discussion of the results relating them to the original research question and placing them into context with the published literature, and conclusions including a discussion of the implications of the research.

Chapter 3 presents the work related to screening, isolation and identification of antagonistic bacteria against *A. euteiches* using *in vitro* assays. Chapter 4 describes the assessment of biocontrol bacteria in growth chamber trials using field pea as a test crop.

The research chapters are followed by a unifying synthesis chapter (Chapter 5) that connects the manuscripts, summarizes the major findings and implications of the research, and highlights the combined contributions of the individual studies. This chapter also includes a conclusions section together with suggestions for further research. Literature cited throughout the thesis are compiled in the Reference section that follows immediately after Chapter 5. Disease score data of the growth chamber trials described in Chapter 4 are included in Appendix A and B, and Appendix C describes nucleotide sequences of the three candidate biocontrol agents.

2. LITERATURE REVIEW

2.1 FIELD PEA PRODUCTION

Field pea *(Pisum sativum* L.) is a grain legume native to southwest Asia and a member of the Leguminosae family. It was among the first crops cultivated by man (Zohary and Hopf, 2002). Since the mid 1980's, Saskatchewan has produced most of the Canadian pea crop although significant production areas occur in Alberta and Manitoba (Spies, 2008). Field peas can fix atmospheric nitrogen when grown in association with appropriate rhizobia and this is one of the advantages field peas has when compared to other non-legume crops. As a result, pea is an important alternative crop which provides rotational benefits.

Field pea is an annual, cool season grain legume that is appreciated for its edible seeds which are rich in dietary protein and energy for humans and livestock (Wang et al., 2011). In western Canada and the Northern USA, varieties with determinate and indeterminate growth habit typically mature between 80 to 90 d and 90 to 100 d, respectively (Njoka, 2008; Spies, 2008). Field pea has a hypogeal emergence and the cotyledons remain below the soil surface following germination. Emergence occurs within 10 to 14 d. The field pea root system is relatively shallow and small, and although 75% of the root biomass is within 60 cm of the soil surface the roots can grow up to a depth of 90 to 120 cm (McKay et al., 2003).

In western Canadian pea production, field pea production in Saskatchewan reached a high of 1.1 million ha in 2005 (Spies, 2008) but more recently production decreased to 867, 000 ha in 2017, which is a reduction of approximately 15% from 2016. This decrease in production was due, in part to the emergence of aphanomyces root rot, although tariffs imposed by India (Statistics

Canada, 2018) also have contributed. Despite the decrease in field pea production area in 2017, Saskatchewan, Alberta and Manitoba accounted for 48%, 50% and 2% of Canadian pea production, respectively (Canadian Grain Commission, 2018). The market destination for most Canadian pea exports between 2008 to 2015 was India, China, Bangladesh, USA and Cuba (Saskatchewan Pulse Growers, 2016).

In western Canada, field pea production is most successful when grown in rotation with cereals, such as barley or spring durum wheat and most pulse crops are resistant to cereal diseases (Agriculture and Agri-Food Canada, 2005). The main role played by field pea as a rotational crop is to provide a break to the build-up of cereal diseases and maintain soil nitrogen fertility (Gregory et al., 2016).

2.2 PEA DISEASES

Seed and seedling, foliar or root diseases in pea plants are caused by either fungi, bacteria, viruses or nematodes (Spies, 2008). These pathogens, under favorable conditions, significantly decrease both crop yield and quality (Wakelin et al., 2002). Examples of common seed and seedling diseases of pea include diseases caused by *Pythium* spp. and *R. solani* (Grünwald et al., 2004). Foliar diseases of pea include white mold, powdery mildew, downy mildew, gray mold, pea rust and Ascochyta blight (Koike et al., 2006). Pathogens such as *A. euteiches*, *Pythium* spp, *Phytophthora* spp and *F. solani* cause root disease in pea (Naqvi, 2007).

Although root diseases damage crops and can result in significant yield losses, root diseases often are less well recognised as they are hidden from view until symptoms are advanced to the upper part of the plant (Wakelin et al., 2002). Since the soil environment is a complex system, root diseases often are difficult to manage, and control or suppression rather than eradication of the

pathogen is normally the goal. Root diseases like aphanomyces root rot are worse as the pathogen can infect at any time in the growing seasons and over the range of temperatures conducive to pea growth, and spores can survive for more than 10 years (Hughes and Grau, 2013).

In western Canada, the most serious root diseases of pea are caused by the pathogens *Aphanomyces* and *Pythium*, both of which belong to a group of fungus-like pathogens referred to as "water mould" (as reviewed in Wu et al., 2018). As the name implies, these pathogens are particularly adapted to wet waterlogged soil. *Pythium* can be controlled with seed treatments (Saskatchewan Pulse Grower, 2017) and there are two recently registered fungicides for an early-season aphanomyces root rot suppression in field pea. These fungicides are INTEGO™ Solo (ethaboxam) and Vibrance® Maxx RFC used with INTEGO seed treatment (Guide for Crop Protection, 2018).

2.3 APHANOMYCES ROOT ROT OF PEAS

Aphanomyces root rot caused by *A. euteiches* is a disease that affects both annual and perennial leguminous plant species including field pea, in different parts of the world (Oyarzun, 1994; Wakelin et al., 2002; McGee et al., 2012; Hughes and Grau, 2013). Although aphanomyces root rot was known to exist in Alberta and Manitoba it was only first reported in Saskatchewan in 2012 (Banniza et al., 2013).

Although field pea is the most notably affected crop, the host range of *A. euteiches* includes some dry bean varieties, lentil, some faba bean varieties and various forage legumes, whereas soybean, chickpea and most faba bean lines are resistant (Vandemark and Porter, 2010). A survey conducted to understand the host range of *A. euteiches* on Canadian cultivars indicated that peas, lentils and cicer milkvetch were the most susceptible crops (Table 2.1) (Chatterton, 2017).

Table 2.1 Result of host range testing of *Aphanomyces euteiches* on Canadian cultivars (adapted from Chatterton, 2017). Canadian cultivars were assessed for disease reaction and presence of oospores after three weeks from infection by *Aphanomyces euteiches* obtained from Saskatchewan and Alberta soils.

Crop	Disease reaction	Oospores
Peas	Susceptible	Yes
Lentils	Susceptible	Yes
Cicer milkvetch	Susceptible	Yes
Dry bean	Variable	Few
Alfalfa	Variable	Yes
Chickpeas	Resistant	Few
Sainfoin	Resistant	Few
Faba bean	Resistant	No
Soybean	Non-host	No
Fenugreek	Non-host	No

2.3.1 Disease symptoms and signs

According to Hughes and Grau (2013) host plants manifest relatively common root rot symptoms when infected by *A. euteiches*. After initial infection, aphanomyces root rot symptom develop within 7 to 14 d and symptom development depends on conditions such as soil moisture, temperature and the concentration of oospores (as reviewed in Wu et al., 2018).

During the primary phase of root rot development, the root system turns soft and water-soaked and turns honey brown or blackish brown (Fig. 2.1) (Hughes and Grau, 2013). At later stages of disease development, symptoms advance from roots into the stems which are usually characterised by yellowing of lower leaves. In extreme cases, dwarfing and death of the entire

plant can occur (Wakelin et al., 2002). Aphanomyces root rot also results in reduction of nodulation (Hwang et al., 2003), which contributes to symptoms of chlorosis (yellowing), and oospores encysted in infected roots are often exposed when roots are cleared and viewed under a microscopic (Fig. 2.1) (Hughes and Grau, 2013). Under field conditions, infected plants appear in patches with coverage ranging from relatively small to high coverage (Clezy, 2016). This is usually associated with poor soil drainage due to soil texture, compaction, and/or over-irrigation as described by Hughes and Grau (2013).

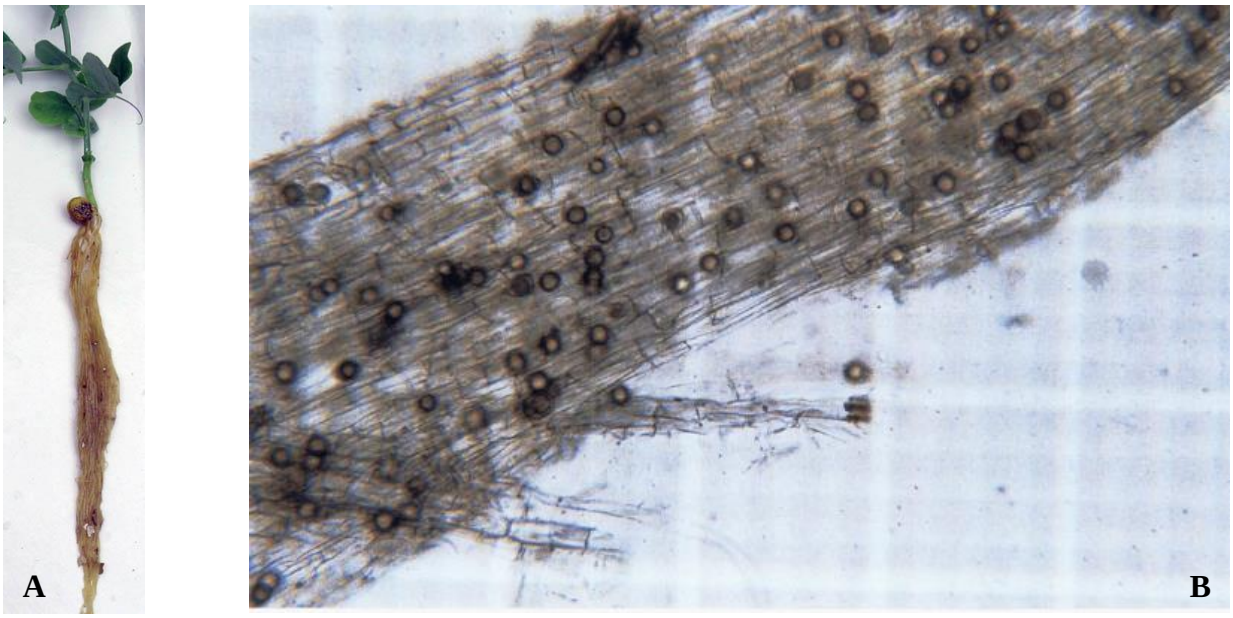

Figure 2.1 Pea roots showing initial lesions, light honey-brown discoloration (A). Oospores of *Aphanomyces euteiches* amongst homogenised pea root tissue on a haemocytometer (B) (Hughes and Grau, 2013).

2.3.2 Disease distribution and severity

Aphanomyces root rot has become a worldwide concern following its occurrence in different pea growing parts of the world (Gaulin et al., 2009). Since it was first reported in 2012 (Banniza et al., 2013) aphanomyces root rot has been an emerging concern in western Canada. As a result, several surveys have been conducted in the Canadian prairies. For example, in 2016 root rot

surveys were conducted across 66 fields in Saskatchewan and Alberta pea fields to assess the presence and severity of root rot pathogens (Clezy, 2016). Results of the surveys indicated that all the pea fields surveyed in Saskatchewan had some root rot, with disease severity ranging from average to moderate across all fields evaluated. Of the fields tested, 44% were confirmed for the presence of aphanomyces. Disease distribution in the fields ranged from appearing as relatively wide coverage to only showing up in patches. Moreover, in some fields, the root rot was not only due to *Aphanomyces* but also involved other pathogens such as *Fusarium*, *Pythium* and *Rhizoctonia*.

2.4 THE GENUS *APHANOMYCES*

Taxonomically, the genus *Aphanomyces* is classified as an oomycete in the Kingdom of diverse eukaryotic protists named Chromista (Hughes and Grau, 2013). Although oomycetes resemble true fungi in some respects, they are distinguished according to some significant differences (Wakelin et al., 2002). For example, oomycetes are diploid for the most part in their lifecycle while true fungi are haploid. Structurally, cellulose and beta-glucans compose oomycetes cell walls whereas the cell wall of true fungi is made of chitin.

The genus *Aphanomyces* comprises three families: *Aphanomyces* plant pathogens; *Aphanomyces* aquatic animal pathogens; and *Aphanomyces* saprophytic species (Gaulin et al., 2018). Among the plant pathogens, *A. euteiches* is the most destructive pathogen that causes root rot disease in many legume plants (as reviewed in Wu et al., 2018). *Bacillus* spp. produces several kinds of antibiotics, including bacillomycin, fengycin mycosubtilin, and zwittermicin, which are effective in controlling the growth of target pathogens (Pal and McSpadden, 2006).

2.4.1 Lifecycle and disease development of *Aphanomyces euteiches*

Aphanomyces euteiches undergoes both sexual and asexual stages in its lifecycle (Fig. 2.2). Oospores, which are the survival stages of *A. euteiches*, range in size from 16 to 25 µm in diameter and have a thick protective wall (Gaulin, et al., 2009). *Aphanomyces euteiches* oospores germinate to mycelia or to zoosporangia when favorable conditions such as susceptible host plant, and warm and moist soil conditions collectively occur (as reviewed in Wu et al., 2018). At the tip of the zoosporangia, clusters of primary zoospores are formed (Wakelin et al., 2002).

Primary zoospores differentiate into secondary zoospores (biflagellate motile zoospores) and they migrate to the host plant in response to chemical signals in the root exudates and encyst quickly on the rhizoplane (Hughes and Grau, 2013). This initial infection is called zoosporic infection (Wakelin et al., 2002). After establishing infection sites, the secondary zoospores germinate into mycelia and produce a structure called a germ tube through which the mycelia extend and infect a new compartment (myceliogenic infection) in the root system and ultimately colonise the entire root system (Hughes and Grau, 2013).

According to Wakelin et al. (2002), while the myceliogenic infection is underway the pathogen produces opposite mating structures called oogonia and antheridia which then form thick-walled oospores within a few days of infection. As the roots decay and break open, oospores are released back to the soil to guarantee long-term survival and cause new infections in subsequent years (Hughes and Grau, 2013).

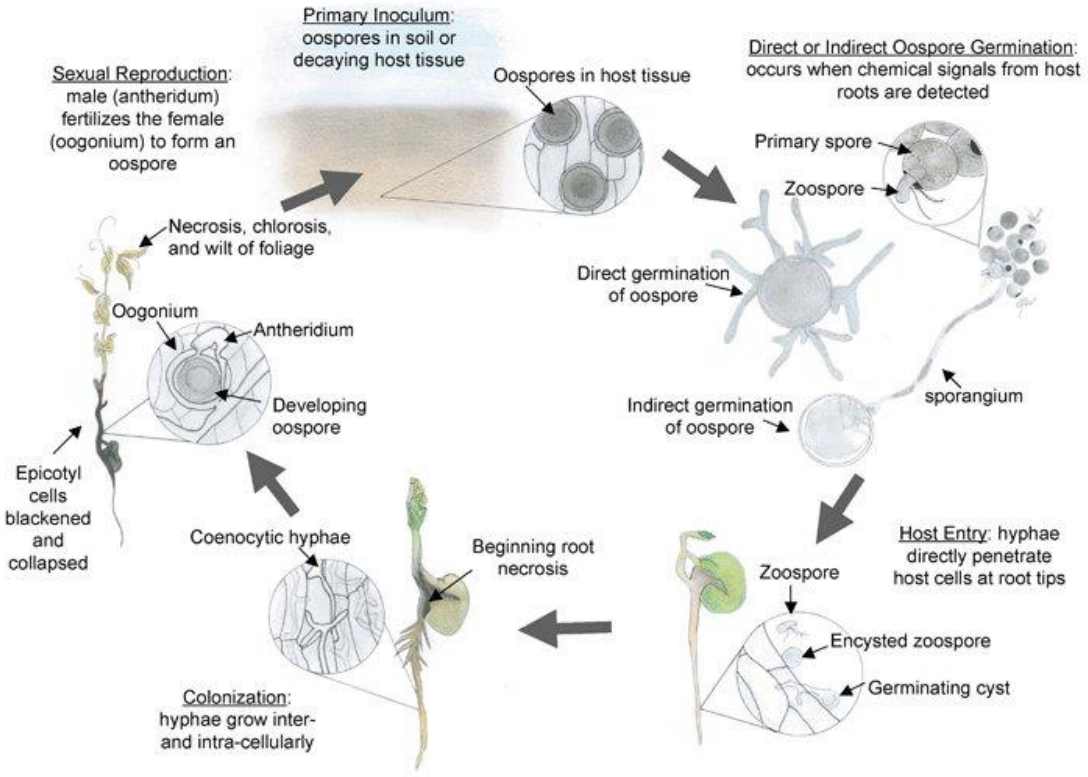

Figure 2.2 Lifecycle of *Aphanomyces euteiches* (Hughes and Grau, 2013).

2.5 FACTORS AFFECTING DISEASE DEVELOPMENT

2.5.1 Pathogen inoculum density

The abundance of a pathogen both in saprophytic and pathogenic stages is referred to as inoculum density and in most cases, it is directly related to the level of disease severity in a susceptible host (Bouhot, 1979). This relationship was also observed between *A. euteiches* inoculum density in soil, and the severity of symptoms root rot disease symptoms in pea (Gangneux et al., 2014). According to Gangneux et al. (2014), root rot severity in pea following infection by *A. euteiches* was highly dependent upon inoculum density of the pathogen.

2.5.2 Temperature

Aphanomyces euteiches can infect field pea throughout the entire range of temperatures that support pea growth; however, the optimum temperature for infection is about 16 °C, and 20 to 28 °C for disease development (as reviewed in Wu et al., 2018). A report by Hughes and Grau (2013) indicated that aphanomyces root rot infection and disease development are exacerbated when seedlings are infected at temperatures ranging from 22 °C to 28 °C. Similarly, Slusarenko (2004) studied the relationships between four temperatures (i.e.,16 °C, 20 °C, 24 °C and 28 °C) and the severity of aphanomyces root rot in field pea. Results of the study indicated that aphanomyces root rot development was favourable at all four temperature regimes evaluated.

2.5.3 Soil moisture

Soil moisture plays a critical role in disease development by influencing germination and migration of flagellated zoospores through the microscopic niches in the soil immediately neighboring the plant roots; thus, the minimum level of soil moisture enough to cause root rot disease is about 30% of the water-holding capacity of the soil (as reviewed in Wu et al., 2018). Compared to other root rot pathogens such as *Rhizoctonia*, *Aphanomyces* is much more water-loving for disease development and spread (Harveson et al., 2014).

2.5.4 Soil type

As described by Persson and Olsson (2000) there are only a few early studies regarding the effect of soil type on aphanomyces root rot. Moreover, there is disagreement about the relationships between soil type and development of aphanomyces root rot. For example, Drechsler (1925) who identified *A. euteiches* for the first time as a causal agent of root rot disease in pea

concluded that any soil that holds water, or in which water was retained due to water-resistant subsoil, could provide conductive conditions for root rot development (Drechsler, 1925). In contrast, Jones and Linford (1925) reported that soil type with different clay content does not affect root rot disease development.

Suppressive soils are soils where a soilborne plant pathogen fails to cause disease despite the presence of a susceptible host and conducive environmental conditions (Chandrashekara et al., 2012). The occurrence of fields suppressive to aphanomyces root rot has led to research in different parts of the world to examine the possible mechanisms by which suppression occurs with the goal of harnessing these mechanisms as control methods. In most cases, suppressiveness is microbially mediated (i.e., antagonizing the pathogen) but in some other cases, soil suppressiveness is due to physicochemical properties of the soil (as reviewed in Wu et al., 2018). For example, studies have shown the involvement of certain clay minerals such as montmorillonite in the suppression of aphanomyces root rot disease (Persson and Olsson, 2000). In addition, calcium concentration and *A. euteiches* are negatively related (Heyman et al., 2007). According to Heyman et al. (2007), aphanomyces root rot development and calcium concentration are observed to have a strong negative correlation and concluded that free Ca is a major variable controlling the degree of soil suppressiveness against *A. euteiches*, and that inhibition of zoospore production from oospores is a possible mechanism.

Survey reports in Saskatchewan indicated that the percentage of pea fields positive for aphanomyces root rot was highest in Black soils followed by Dark Brown and Brown soils (Chatterton et al., 2017), suggesting the possible influence of soil organic matter and/or moisture.

2.5.5 Interaction with other pathogens

Root rots of pea in the field are complex diseases that are caused by root rot complex, including pathogens such as *Pythium*, *Fusarium* and *Rhizoctonia*. In such cases, identification of the primary pathogen and the role of each pathogen and the nature of the interrelationships between pathogens is difficult to ascertain (Hughes and Grau, 2013). There is an increased risk of yield loss in regions where *A. euteiches* and other pathogens, for example, *Fusarium* spp. co-occur (as reviewed in Wu et al., 2018). Similarly, Willsey et al. (2018) observed higher disease severity in the presence of root rot complex pathogens than when a single species was present. This observation also was confirmed by qPCR analysis which revealed extensive colonization in treatments involving multiple species.

2.6 CONTROL OF APHANOMYCES ROOT ROT OF PEA

Among the pathogens causing root rot in field pea, *A. euteiches* is now recognized as the most damaging species (as reviewed in Wu et al., 2018). Furthermore, options for management are limited and completely resistant pea cultivars are absent (Conner et al., 2013; Lavaud et al., 2015). Currently, the approach that is widely advised is lengthening the frequency of planting susceptible crops by crop rotation and identification of the pathogen inoculum level in the fields prior to planting (Hughes and Grau, 2013).

2.6.1 Chemical control

Some chemicals which are active against other oomycetes are not active against *A. euteiches* (Wakelin et al., 2002). Nevertheless, few chemical controls have been reported to suppress this pathogen under controlled conditions with limited useful effect in field experiments (Xue, 2003).

At present, INTEGO™ Solo (ethaboxam) and Vibrance® Maxx RFC are two recently registered fungicides for suppression of an early-season aphanomyces root rot in field pea in Canada (Guide for Crop Protection, 2018).

2.6.2 Cultural control

Crop rotation is the oldest method used by farmers to protect crops from diseases like aphanomyces root rot and for the most part, its effectiveness relies on the length of rotational intervals between susceptible and resistant crops (as reviewed in Wu et al., 2018). Farmers typically use crop rotation to control the rate of the build-up of pathogenic inoculum in soils; however, crop rotation as an approach to manage aphanomyces root rot in pea is questionable because *Aphanomyces* oospores are long-lived and can stay in the soil for more than 10 years under unfavorable conditions (Hughes and Grau, 2013) and the presence of other alternative host plants such alfalfa, lentils, cicer milkvetch and some dry bean varieties (Chatterton, 2016), and even weedy plant species (Papavizas and Ayers, 1974) can prolong the presence of the disease organism.

Currently, the recommended rotation cropping interval for field pea is between six to eight years (Hughes and Grau, 2013; Hossain et al., 2014). To improve the efficiency of crop rotation it is advisable to increase crop diversity (Krupinsky et al., 2002).

2.6.3 Disease avoidance

According to Wakelin et al. (2002), disease avoidance is a method that involves the indexing of field soil to determine the inoculum potential of *A. euteiches* or infestation level of soil prior to growing a susceptible crop. The indexing assay of field soil involves soil sampling from a test

field, mixing the samples and growing peas in the soil mix under controlled conditions that are disease conducive. Initially, plants are removed after several weeks of growth and roots are washed, the roots are assessed visually for disease, and a disease index score is determined. Finally, based on the disease index score results, the site is categorised as a hazardous field, nonhazardous field or slightly-infested field, and appropriate management decisions can be made (Moussart et al., 2006; Sauvage et al., 2007)

2.6.4 Host resistance

A great deal of time has been invested in breeding pea cultivars which are genetically resistant to *A. euteiches*. Despite the absence of high-level resistance within the species, several pea breeding lines with partial resistance to tolerance have been developed (Conner et al., 2013).

Conner et al. (2013) identified a good level of tolerance in pea line 00-2067 after conducting a comparative study between a disease-free site and an aphanomyces root rot site in Manitoba, Canada. Furthermore, the report indicated that in pea line 00-2067 a higher plant vigor and yield was consistently recorded in all sites. Similarly, Wu (2018) obtained comparable results; thus, this pea line might be a candidate for future advanced agronomic studies. Even though challenges such as pathogenic variability within *A. euteiches* and difficulties in breeding resistant pea lines exist, findings by Conner et al. (2013) and Wu (2018) are the bright light in the process of developing tolerant and/or resistant pea cultivars.

2.6.5 Biological control

Plant pathologists define biological control (biocontrol) as the application of microorganisms for diseases management and the use of host-specific pathogens to control weed

populations. Such organisms are referred to as biological control agents or biocontrol agents (Wakelin et al., 2002). In its broad sense, biocontrol encompasses the use of natural products obtained by fermentation or other methods (Singh et al., 2016).

Biological control agents applied to seeds or fields in various formulations may protect pea crops from pathogens such as *A. euteiches*. For example, research by Wakelin et al. (2002) identified spore-forming bacteria that substantially supressed aphanomyces root rot in pea when used as a seed treatment both under greenhouse and field conditions. The natural compound isothiocyanate which is produced by plants of the Brassicaceae family is reported to have toxic effects towards *A. euteiches* and hence is used for the management of aphanomyces root rot under controlled conditions (Hossain et al., 2014).

Soil bacterial communities and their interactions are diverse and complex both at interspecies and intraspecies levels and have the capacity to respond to environmental variability (Mauchline and Malone, 2017). Soil microbes are adapted to live in close association with plant root systems and some act as plant growth-promoting rhizobacteria (PGPR). These organisms have a positive impact on plant growth and development through the production of microbial siderophores, antibiotics, solubilization of elements (biofertilizing) and phytohormones, competition for space and nutrients, induced systemic resistance and quorum quenching (Bienkowski, 2012). Rhizosphere bacteria may suppress aphanomyces root rot diseases by disrupting the growth of the pathogen on field pea root tissues, suppressing the formation of mycelia and zoospore infection structures and stimulating plant defences. Therefore, manipulation of soil microbes and their bioproducts for the control of the soil born pathogens *A. euteiches* holds great promise and may compliment other forms of control.

3. SCREENING, ISOLATION AND IDENTIFICATION OF ANTAGONISTIC BACTERIA AGAINST *APHANOMYCES EUTEICHES* USING *IN VITRO* ASSAYS

3.1 ABSTRACT

Aphanomyces euteiches is a soil born pathogen that causes root rot of pea and could significantly affect the sustainability of pea production in western Canada. Zoospore and mycelia are the infective stages primarily responsible for the development of aphanomyces root rot symptoms. The aim of this study was to isolates and identify soil bacteria with biocontrol effect towards *A. euteiches* mycelia and zoospore developmental stages under laboratory conditions.

Initial screening of soil bacteria *in vitro* identified 184 isolates antagonistic to *A. euteiches* mycelia growth, of which 22 were from a previously existing bacterial culture collection. Further screening of these antagonistic bacteria identified 47 isolates that inhibited *A. euteiches* zoospore germination by 75% or more compared to control plates. The mean mycelial growth inhibition zone ranged from 1 to 12 mm. The maximum inhibition zone was recorded in treatment involving isolate K-CB2-4 (*Lysobacter antibioticus*). The mean zoospore germination inhibition ranged from 0 to 100 % for stock and 100-fold dilutions. Isolate K-Hf-L9 (*Pseudomonas fluorescens*) completely inhibited zoospore germination when applied as a stock solution and when diluted 100-fold. Based on molecular data, the antagonistic bacterial isolates were placed into 18 different genera with *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Lysobacter* and *Streptomyces* being the top five genera with large numbers of isolates. Variations of inhibition potential among isolates suggests that the mechanisms by which biocontrol is achieved likely varies between isolates. The identification of antagonistic bacteria suggests that the pathogen lifecycle can be interrupted, and there is a potential promise to control or reduce aphanomyces root rot using biocontrol organisms.

3.2 INTRODUCTION

Aphanomyces root rot of pea is becoming an economically important disease in Canada and in most pea growing areas of the world (Oyarzun, 1994; Wakelin et al., 2002; McGee et al., 2012; Hughes and Grau, 2013). The disease is caused by an oomycete pathogen, *A. euteiches* and it has two infective stages called zoospores and mycelia which are capable of infecting pea plants at any growing stage (Hughes and Grau, 2013). Moreover, the pathogen has survival structure known as oospores that can live for more than 10 years under unfavorable conditions (Hughes and Grau, 2013; Wu et al., 2018). Aphanomyces root rot symptoms can be grouped as below-ground (early stage symptoms) and above-ground (late stage symptoms). Below-ground symptoms include the production of water-soaked, honey-coloured lesions on the root tissue, and roots eventually become soft honey-brown or blackish-brown in appearance with reduced size and function. Above-ground symptoms include yellowing and wilting of lower leaves, dwarfing and stunting of plants, and even death of the entire plant (Wakelin et al., 2002; Hughes and Grau, 2013). In infested soil, loss of productivity due to this pathogen can reach up to 100% (Gaulin et al., 2007). In addition, options for management are limited, and completely resistant pea cultivars are absent (Conner et al., 2013; Lavaud et al., 2015). Currently, the approach that is widely advised is lengthening the rotation of planting susceptible crops and identification of the pathogen inoculum level in the fields prior to planting. In Canada INTEGO™ Solo (ethaboxam) and Vibrance® Maxx RFC were recently registered fungicides for the suppression of early season aphanomyces root rot in field pea (Guide for Crop Protection, 2018). As a result, there is still a need to develop effective control methods, including the development of biological control agents.

When considering the development of a biological control agent against aphanomyces root rot in field pea, it is useful to target infective stages within the lifecycle of the pathogen, as

interruption of these developmental phases may ultimately provide protection to the plant. Another important consideration is the selection of the type of organism used as a biocontrol agent. Ideally the microorganisms selected should be naturally present and presumably adapted to the soil immediately surrounding the pea root (i.e., rhizosphere soil). Such consideration will increase the chance of finding isolates adapted to the pea rhizosphere environment where *A. euteiches* occurs.

The rhizosphere is a complex environment at the interface between soil and plant roots (Dessaux et al., 2016). This region is active both chemically and biologically, hence it plays a pivotal role in the process of the development of diverse microbial communities (Cavaglieri et al., 2009). These diverse microbial communities are influenced by environmental parameters and parameters related to the physiochemical properties of the soil. Moreover, biological activities of plants, chemical signals from root exudates and microbes which inhabit soil adherent to root-system, continuously influence the diversity of microbial communities (Haldar, and Sengupta, 2015). As a result, this region is perceived as a hot spot of biodiversity (Yadav et al., 2017).

Plants release a considerable amount (i.e., up to 40%) of the fixed carbon through root exudation and as a result the rhizosphere region is rich in nutrient composition compared to the bulk soil (Jia et al., 2015). Therefore, different microorganisms have developed distinct strategies such as neutralism, commensalism, synergism, mutualism, amensalism and antagonism for survival and fostering community development in the rhizosphere (Montesinos, 2003). The rhizosphere microbiome is largely dominated by fungi and bacteria, and generally the number of bacteria that inhabit this region is 10 to 100 times higher than in the bulk soil (Adriano et al., 2005).

Rhizosphere bacteria benefit plants in many ways, one of which is antagonizing pathogens (Han et al., 2005). The mechanisms by which bacteria antagonize plant pathogens include

competition for nutrients and space, production of antibiotics and toxins, or production of host cell wall degrading enzymes (Krechel et al., 2002). Bacteria with such antagonistic attributes have the potential to be developed into biological control agents for the management of various crop diseases including aphanomyces root rot disease of field pea (Khabbaz and Abbasi, 2013). Therefore, screening, isolation and detection of bacteria antagonistic towards a known plant pathogen is an integral part of biological control agent development.

The use of selective procedures that allow the detection and isolation of only those microorganisms of interest from a large microbial population is called screening (Srividya et al., 2008). Different screening approaches have been employed extensively in the search for microorganisms capable of producing useful antibiotics. One of these techniques is a "crowded plate" procedure (Chandrashekhara et al., 2010). This technique has been modified by the incorporation of a known microorganism (test organism) that is used as an indicator organism for the presence of specific antagonistic bacteria (Kelner, 1948).

Primary screening of biological control agents involving a host plant is not feasible due to the diversity of agents and interactions with the host plant (Mota et al., 2017). As a result, an effective screening method that has a high probability of detecting microbes at a low cost is required. In recent years, it has become a common practice to conduct initial screening procedures in the absence of the host plant (Validov et al., 2007; Köhl et al., 2011). In this regard, due to the increased likelihood of screening a high number of antagonistic bacteria from a large background of soil microbes and the low cost of the procedure, *in vitro* assays may be suitable for screening of bacteria possessing antagonistic attributes to *A. euteiches*. Therefore, the objectives of the study were: 1) to isolate bacteria possessing antagonistic effect against *A. euteiches*; 2) to screen *A. euteiches* mycelia growth inhibitory bacteria; 3) to screen *A. euteiches* zoospore germination

inhibitor bacteria and 4) to determine the identity of the antagonistic bacteria using molecular techniques.

3.3 MATERIALS AND METHODS

3.3.1 Soil sampling and processing

Soil sampling was conducted in 2016 during the periods of active pea growth from 43 pea fields across Saskatchewan (Fig. 3.1). Where possible, soils were sampled from areas of the field exhibiting disease symptoms, and from apparently healthy areas of the field [i.e., potentially suppressive soils as described by Chandrashekara et al. (2012)]. In each field, one 40 m transect was established and four points, each of which was 10 m apart along the transect, served as locations for soil sampling. The soil samples were collected from with in the seedrow to a depth of 15 cm from each sampling point using a soil probe (3 cm diameter). The specific location of each sampling point was recorded using a global positioning system (GPS). All soils were stored on ice upon collection and transported to the University of Saskatchewan Soil Science Laboratory for microbial isolation. Immediately, the field moist samples were sieved (2 mm) and the fine soils were stored in 111 mL snap cap vials at -20 °C for bacterial isolation. Additionally, a portion of each soil sample was air-dried to determine soil pH and soil organic carbon (SOC). Soil pH (1:2 soil suspension; soil:water) (Kalra and Bhatti, 2006) was measured using a Beckman 50 pH Meter (Beckman Coulter, Fullerton, CA). Following HCl treatment to remove carbonates (Wang and Anderson, 1998), soil organic carbon was determined using a LECO C632 Carbon Analyzer Leco (Corporation, St. Joseph, MI).

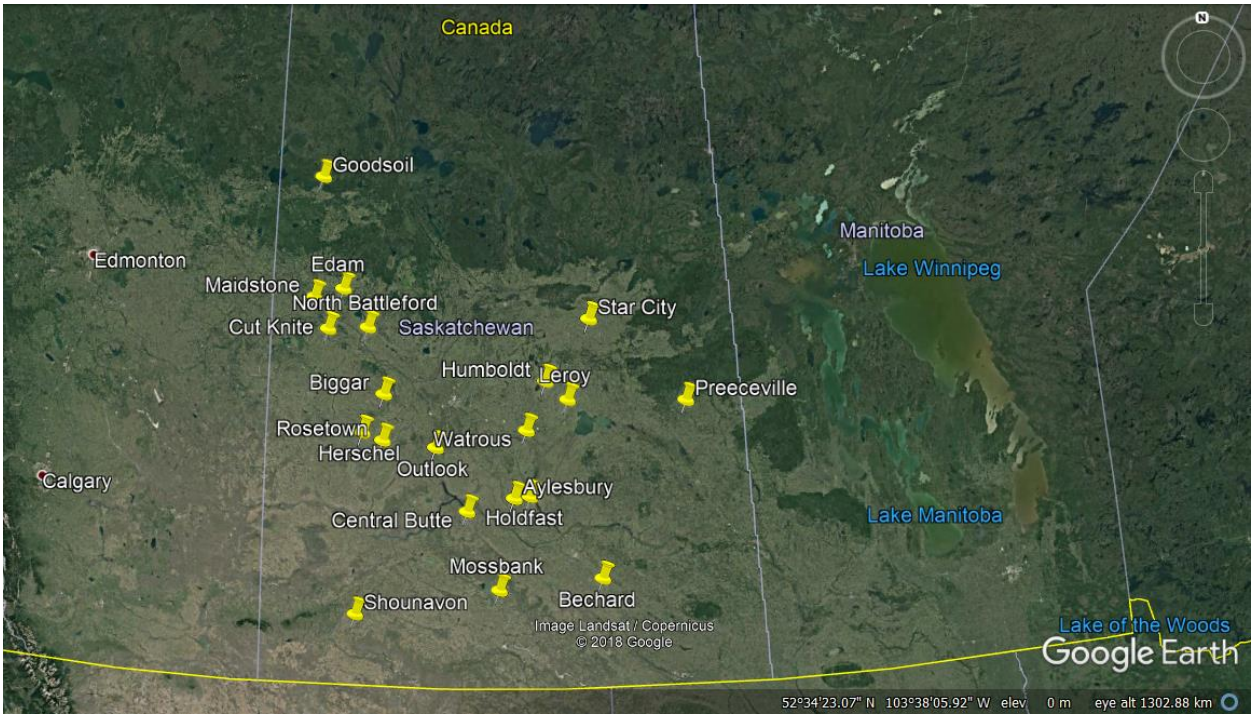

Figure 3.1 Google Earth map showing soil sampling sites. The yellow pins show the specific locations of the sampling sites in Saskatchewan, Canada.

3.3.2 Determination of dilution with viable bacterial cell count

The soil samples initially stored at -20 °C storage were brought to room temperature over a period of 14 h and 5 g of soil from each sample subsequently was mixed with 45 mL of sterile phosphate buffer saline (PBS) in a sterile 50 mL centrifuge tube. This was shaken at 150 rpm for 25 min and then centrifuged at 2000 rpm for 1 min to remove coarse particles. The supernatant was transferred to sterile 50 mL Falcon tubes (Falcon, Corning Science Mexico S. A. de C.V., Tamaulipas, Mexico).

A serial dilution (10^{-1} to 10^{-5}) was prepared in PBS by transferring 1 mL of slurry into 9 mL of PBS. Aliquots of 0.1 mL were pipetted from each dilution and spread on 1/10 strength trypticase soy agar (TSA) plates. Enumeration of viable bacteria colony forming units (CFU) was determined after incubating the plates at 28 °C for 72 h.

3.3.3 Primary screening of *Aphanomyces euteiches* antagonistic bacteria

Screening of soil bacteria possessing antagonistic properties against the mycelial development stage of the *A. euteiches* lifecycle was done using a modified crowded plate assay (Waksman, 1945). Serial dilutions of soil samples were prepared in PBS based on the previous viable bacterial cell count. Appropriate dilutions with countable colonies (10^{-2} to 10^{-4}) were spread over fresh 1/10 TSA (150 x 25 mm) plates. These plates were allowed to dry for 4 h in a laminar-flow hood to allow the establishment of the soil bacteria on the plate surface.

After the initial drying time, 0.75 mL of crushed *A. euteiches* mycelia were pipetted and spread on the surface of the assay plates. The *A. euteiches* inoculum was prepared from a pure culture of *A. euteiches* (AE1) that was obtained from the University of Saskatchewan Plant Science-Crop Development Centre (courtesy Dr. Sabine Banniza, University of Saskatchewan). Initially, four plugs of *A. euteiches* mycelia (5 mm) were taken from a PDA mother plate that had been incubated for 3 d to develop a mycelial mat and transferred into a 500 ml flask containing 200 mL potato dextrose broth, and thirty 2.8 mm ceramic beads (Omni International, USA) and a magnetic stirring bar. The broth culture was incubated at 23 °C and 120 rpm for 5 d under dark conditions. Finally, the mycelia were crushed on a magnetic stirrer at 350 rpm for 25 to 30 min.

Following inoculation with *A. euteiches*, assay plates were inverted and incubated for 5 d at 23 °C under dark conditions. The plates were inspected visually and antagonistic bacteria inhibiting mycelial growth and having a clearing zone around them were identified (Fig. 3.2). Antagonistic bacteria were isolated and subsequently cultured in half strength trypticase soy broth (TSB) for 3 d and stored at -80 °C as a glycerol stock for later use in this experiment. Briefly, the

glycerol stock cultures were prepared by mixing 1 mL of bacterial inoculum with 1 mL of glycerol as described in Altermatt et al. (2015).

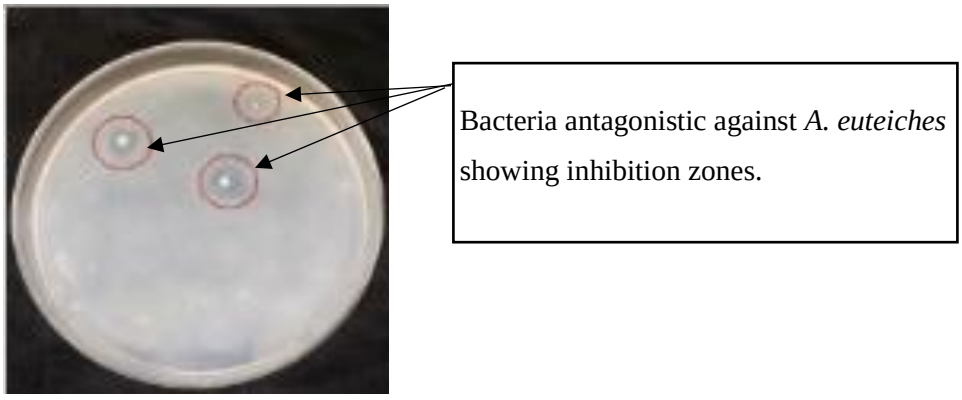

Figure 3.2 Primary screening of antagonistic bacteria using a modified crowded plate assay method.

3.3.4 *Aphanomyces euteiches* mycelial growth inhibition assay

Glycerol stock cultures of the previously identified antagonistic bacteria were taken from -80 °C storage and allowed to thaw at room temperature for approximately 10 min. A loopful of each glycerol stock culture was subsequently streaked on fresh 1/10 TSA plates and the plates were incubated at 28 °C for 72 h. After incubation, a single colony was streaked along two opposite edges, 1.5 cm away from the periphery of a fresh potato dextrose agar (PDA) plate (Xu and Kim, 2014).

Following inoculation of the PDA plates with antagonistic bacteria, a plug of *A. euteiches* (5 mm diameter) was taken from 3 d PDA culture using a sterile metal corer and placed at the center of the assay plates. The plates were incubated for 5 d at 23 °C in the dark. The inhibition zone (i.e., the clearing zone in the interface between the tip of the mycelia and the colony edge) was measured at six interaction zones from three assay plates as described in Wakelin et al. (2002). Briefly, after 5 d of incubation, the measurement was taken between the front edge of the colony facing the original *A. euteiches* plug placed at the center and the tip of the mycelia inhibited. *Aphanomyces*

euteiches mycelia were considered to be "inhibited" if no growth was detected up to or past the point of bacterium inoculation (Fig. 3.3). Furthermore, a preliminary screening assay utilized a dual plate technique was to assess 170 bacterial isolates for biocontrol activity against *A. euteiches*. These 170 bacterial isolates were from an existing bacteria culture collection of root associated rhizobacteria from a variety of field crops (courtesy Dr. J. Germida, University of Saskatchewan).

Figure 3.3 *Aphanomyces euteiches* mycelial growth inhibition assay on potato dextrose agar (PDA).

3.3.5 *Aphanomyces euteiches* zoospore germination inhibition assay

Antagonistic bacterial isolates that inhibited mycelia growth were further assayed for zoospore germination inhibition potential. *Aphanomyces euteiches* zoospores were produced based on Islam et al. (2007) and modified by the Saskatchewan University Plant Science-Crop, Development Centre (pers. comm., Dr. Sabine Banniza). Four plugs of *A. euteiches* (5 mm) were taken from 5% Corn Meal Agar (CMA) mother plates that had been incubated for 3 d to develop a mycelial mat and transferred to CYP agar (CMA + Yeast Extract + Phosphate Buffer) plates on which approximately 15 4-cm-long autoclaved wheat leaves were placed on the surface (Fig. 3.4). These plates were incubated for 4 d at 23 °C under dark conditions. On the fourth day, *A. euteiches* mycelia with the wheat leaves were transferred to 100 mL sterile distilled water in a 250 mL flask. The flasks were covered with tinfoil and placed on a shaker for 16 h at 110 rpm to induce *A.*

euteiches zoospores. Target zoospore concentration was adjusted by using a hemocytometer to determine zoospore populations and diluting with sterile distilled water, as required.

Figure 3.4 Corn Yeast Phosphate agar (CYP agar) (5% Corn meal Agar + Yeast Extract + Phosphate Buffer) with autoclaved wheat leaves.

The antagonistic bacteria were cultured for 24 h in 10 mL of half strength TSB. This broth culture served as bacterial stock dilutions. For each bacterial isolate, a 100-fold dilution of the stock solution was prepared in PBS. Aliquots of 0.1 mL of both dilutions (stock and 100-fold) were spread over two PDA plates and after 4 h of pre-colonization time, 0.75 mL of *A. euteiches* zoospores (0.5×10^4 zoospores mL^{-1}) were pipetted and spread over the PDA plates. These assay plates were incubated at 23 °C for 3 d under dark conditions. After 3 d, four fields of the assay plates were observed under a compound microscope with 100X total magnification for inhibition of zoospore germination. A zoospore was considered to be germinated when active motile zoospores were observed.

The degree to which zoospores were germinated was assessed according to Wakelin et al. (2002). Briefly, the data was collected based on the level of germination on four random fields on the assay plates. Thus, germination score data were recorded as follows: 0 = no germination; 1= light germination (i.e., less than 33% germination); 2 = medium germination (i.e., between 33% and 67% germination); and 3 = heavy germination (i.e., more than 67% germination) (Fig. 3.5). For each isolate, a total score out of a possible 24 (2 plates X 4 observations X maximum score 3

according to the scoring protocol) was calculated. Finally, the results were converted to zoospore germination inhibition scores and expressed as percentages. Plates consisting of zoospores and 0.1 mL of TSB served as a positive control whereas plates consisting of 0.1 mL of TSB were considered a negative control.

Zoospore germination was calculated according to Wakelin et al., (2002) as follows:

Zoospore Germination Score % = (((O1 + O2 + O3 + O4) *n) *100)/N (Eq. 3.1)

Based on the zoospore germination score, zoospore germination inhibition score was calculated to determine the antagonistic potential of the isolates in terms of percent inhibition compared to a positive control as follows:

Zoospore Germination Inhibition Score % = (100 - Zoospore Germination Score %) (Eq. 3.2)

where *O* is germination score at each observation in each assay plates, *N* is the total possible score (i.e., 24), and *n* is the number of plates for each dilution (i.e., 2).

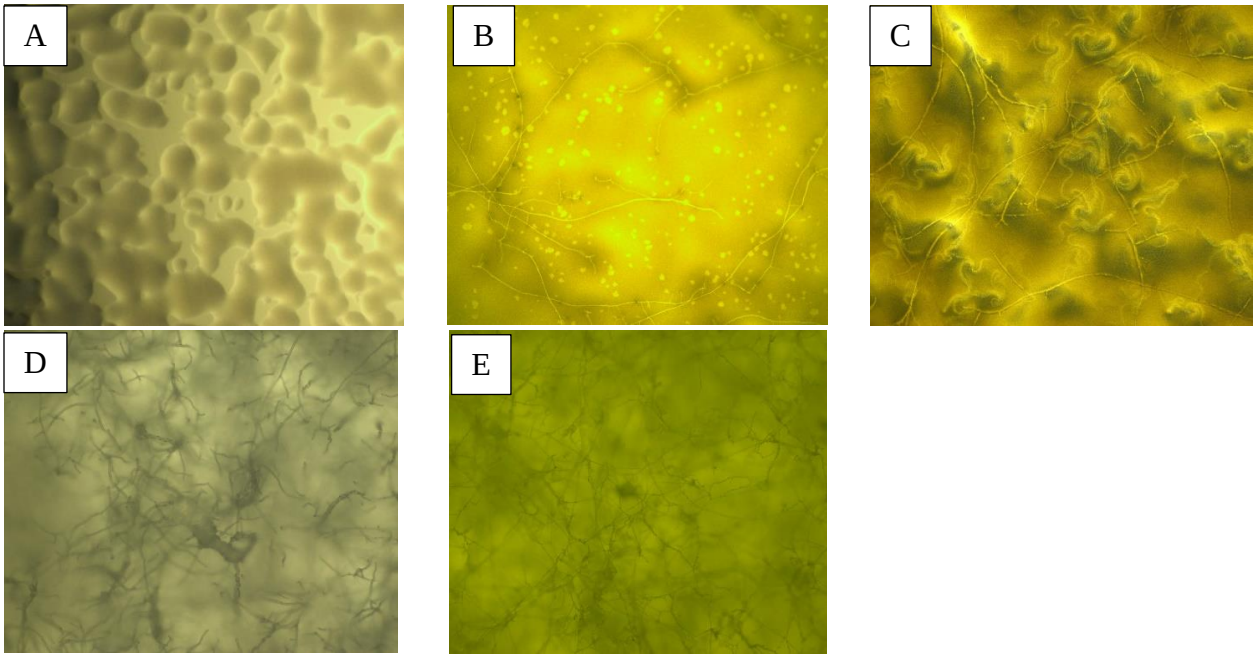

Figure 3.5 *Aphanomyces euteiches* zoospore germination inhibition scoring protocol, based on the degree of hyphal development. A = No zoospore germination (0), B = light (1), C= medium (2) and D = Heavy (3). Field "E" was the positive control to which all the assay plates were compared.

3.3.6 Data collection and analysis

Correlation analysis was performed to determine whether pH, SOC and total heterotrophic bacterial count were quantitatively related. Thus, Pearson correlation coefficients (r) were determined using SAS computer package, Pearson Correlation, Prob > |r| under H0: Rho=0 (SAS, 9.3).

Aphanomyces euteiches mycelia growth inhibition data were collected from six interaction zones and the isolates were transferred to zoospore germination inhibition assay. *Aphanomyces euteiches* zoospore germination inhibition scores were determined using a 0 to 3 scale, where 0 = no germination; 1 = light germination; 2 = medium germination; and 3 = heavy germination. Scores were a total of eight observations (microscopy fields) from two assay plates for the stock and 100-fold bacterial culture dilution. The positive controls were PDA assay plates consisting of *A. euteiches* zoospore challenged with autoclaved distilled water. The antagonistic potential of each isolate was ranked based on the zoospore germination inhibition scores and isolates that inhibited zoospore germination by 75% or more were selected for growth chamber trials.

Correlation analysis was performed to determine whether mycelia inhibition and zoospore germination inhibition at stock concentration and 100-fold dilution of the antagonistic bacteria were quantitatively related. Thus, Pearson correlation coefficient (r) was performed using SAS computer package, Pearson Correlation, Prob > |r| under H0: Rho=0 (SAS, 9.3).

3.3.7 Molecular identification of antagonistic bacterial isolates

The identity of all putative biocontrol bacteria obtained in the screening stage was identified using molecular techniques. Glycerol stock samples (200 µL) of each bacterial isolate in a 96-well

microtiter plate was submitted for analyses to Génome Québec Innovation Centre (McGill University, Quebec).

Amplification of the 16S rDNA gene was conducted using the primer 27F (5' AGAGTTTGATCMTGGCTCAG 3')/1492R (5' TACGGYTACCTTGTTACGACTT 3') and the amplified PCR product was analyzed using Sanger sequencing (Ye et al., 2009).

Nucleotide alignments of the antagonistic bacterial isolates' DNA sequences were carried out using CLUSTAL W program from the MEGA software packages (Kumar et al., 2016) and the same software was used to build maximum likelihood trees using Kimura's 2-parameters distance correction. The robustness of the tree topology was calculated from bootstrap analyses with 100 replications. Sequence similarity was calculated from each gene and genospecies using pair-wise sequence alignments by Kimura 2-parameters model, as implemented in MEGA version 7 (Kumar et al. 2016).

The evolutionary history was inferred using the Maximum Likelihood method (Kumar et al. 2016). Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair-wise sequence alignments using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log-likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 112 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. These evolutionary analyses were conducted in MEGA version 7 (Kumar et al. 2016).

3.4 RESULTS

3.4.1 Soil pH, organic matter content and total heterotrophic bacteria

The 43 soil samples (Fig. 3.6) had a wide range of values for pH, organic carbon content and total heterotrophic bacteria (Table 3.1). For example, the soil pH ranged from 5.0 to 8.5, that is, acid to alkaline and the values were location dependent. Organic carbon contents of the soil samples ranged from 8.8 mg g^{-1} to 52.2 mg g^{-1}. Total heterotrophic bacteria in the sampling locations ranged from 5.1 x 10^6 CFU g^{-1} to 7.7 x 10^8 CFU g^{-1}. The wide range of pH, organic matter content and total heterotrophic bacteria provided ample opportunity to explore the relationships between soil pH, organic matter content and total heterotrophic bacteria (Table 3.2).

Figure 3.6 Air-dried soil samples used to determine soil pH and soil organic carbon (SOC).

Table 3.1 Soil pH, soil organic carbon (SOC) and total heterotrophic bacteria at the sampling locations in Saskatchewan.

S.N.	Sampling locations	Soil analysis		Total heterotrophic bacteria (g^{-1} soil)
		pH	SOC (mg g^{-1} dry soil)	
1	Aylesbury high spot	6.3	26.4	1.8×10^8
2	Aylesbury low spot	6.1	31.6	7.7×10^7
3	Bechard high spot	7.8	24.2	2.3×10^8
4	Bechard low spot	8.3	22.6	6.8×10^8
5	Biggar high spot	6.9	28.3	6.4×10^7
6	Biggar low spot	6.9	29.7	4.9×10^8
7	Central Butte (symptom of root rot)	8.0	10.4	1.7×10^7
8	Central Butte (no symptom root rot)	7.8	9.6	1.2×10^8
9	Cut Knite (symptom of root rot)	6.5	24.9	9.5×10^7
10	Cut Knite (no root rot symptom)	6.0	23.8	1.7×10^7
11	Drake Leroy (symptom of root rot)	7.8	24.2	5.8×10^6
12	Drake Leroy (no root rot symptom)	7.9	11.8	1.3×10^7
13	Edam (symptom of root rot)	6.9	8.8	1.2×10^8
14	Edam (no root rot symptom)	6.7	16.0	5.1×10^6
15	Goodsoil high spot	5.0	22.6	5.2×10^7
16	Goodsoil low spot	7.8	52.1	3.2×10^8
17	Herschel high spot	7.3	27.4	1.4×10^8
18	Herschel low spot	7.5	27.2	7.2×10^8
19	Hold fast high spot	8.0	25.2	1.4×10^8
20	Hold Fast low spot	7.5	35.4	1.4×10^8
21	Humboldt (symptom of root rot)	8.4	19.4	1.6×10^7
22	Humboldt (no root rot symptom)	8.5	21.9	5.1×10^6
23	Humboldt high spot	8.2	18.6	1.1×10^8
24	Humboldt low spot	8.0	19.6	6.5×10^8
25	Leroy (symptom of root rot)	8.3	17.9	1.4×10^8
26	Leroy (no root rot symptom)	8.2	24.1	1.3×10^7
27	Maidstone (no symptom of root rot)	8.0	13.6	4.7×10^7
28	Maidstone (symptom of root rot)	7.5	30.3	9.7×10^7

Continued

S.N.	Sampling locations	Soil analysis		Total heterotrophic bacteria (g^{-1} soil)
		pH	SOC (mg g^{-1} dry soil)	
29	Mossbank high spot	8.0	17.7	7.3×10^7
30	Mossbank low spot	7.6	36.0	7.7×10^8
31	North Battleford (symptom of root rot)	7.0	12.9	3.8×10^7
32	North Battleford (no root rot symptom)	6.4	13.6	1.3×10^7
33	Outlook (symptom of root rot)	7.6	14.5	7.4×10^7
34	Preeceville high spot	7.9	25.4	3.2×10^8
35	Preeceville low spot	8.0	52.2	1.4×10^8
36	Rosetown high spot	8.2	25.9	1.4×10^8
37	Rosetown low spot	8.3	20.6	5.9×10^8
38	Shaunavon high spot	7.6	11.7	1.6×10^7
39	Shaunavon low spot	7.8	18.1	3.5×10^8
40	Star City high spot	6.3	38.0	4.8×10^8
41	Star City low spot	6.1	43.1	3.6×10^8
42	Watrous (symptom of root rot)	7.6	24.2	1.5×10^7
43	Watrous (no root rot symptom)	7.7	21.0	1.9×10^7

Correlation analysis showed that soil pH had no significant correlation with SOC ($p = 0.32$) and total heterotrophic counts ($p = 0.54$) across the sampling locations. However, a significant correlation ($p = 0.02$) was found between SOC and total heterotrophic bacteria (Table 3.2).

Table 3.2 Pearson correlation coefficient (r) analysis between pH, soil organic carbon (SOC) and total heterotrophic bacteria.

	pH	SOC	Total heterotrophic bacteria †
pH	1	-0.15	-0.16
SOC		1	0.14*
Total heterotrophic bacteria			1

† Correlation analysis was performed to determine whether pH, SOC and total heterotrophic bacterial count were related. Thus, Pearson correlation coefficient (r) was performed using SAS computer package. Pearson Correlation Coefficients, Prob > |r| under H0: Rho=0. * denote p values ≤ 0.05.

3.4.2 Primary screening of antagonistic bacteria

A modified crowded plate assay was used to identify bacteria antagonistic to *A. euteiches*. From the 43 Saskatchewan soil samples obtained from fields in which field pea predominantly had been grown, 162 antagonistic bacteria were identified that inhibited mycelia stage of *A. euteiches* (Table 3.3).

3.4.3 Mycelia growth inhibition assay

For comparative analysis of antagonistic potential, all isolates identified using the modified crowded plate assay were further assayed for potential biocontrol characteristics using a dual plate assay, which also identified 22 antagonistic bacteria from a previously existing bacteria culture collection (Table 3.3). The antagonistic bacterial isolates inhibited *A. euteiches* mycelial growth to varying degrees, ranging from a minimum zone of inhibition of 1 mm to a maximum zone of inhibition of 12 mm (Table 3.3, Fig. 3.7). Isolates such as K-CB2-4, K-CB2-2, K-CB2-3, K-CB2-1, K-MB-H5, K-SC-L2 and K-Be-H3 had the highest zone of inhibition.

Table 3.3 *Aphanomyces euteiches* mycelia growth inhibition by antagonistic bacterial isolates *in vitro* assay.

Serial No.	Isolate	Mean inhibition zone (mm)	Serial No.	Isolate	Mean inhibition zone (mm)	Serial No.	Isolate	Mean inhibition zone (mm) †
1	K-CB2-4	12	36	E3-1	4	71	K-Hf-L7	3
2	K-CB2-2	11	37	Ler4-2	4	72	K-O4-1	3
3	K-CB2-3	11	38	K-Hf-H1	4	73	K-BG-H4	3
4	K-CB2-1	11	39	DR1-3	4	74	K-MS4-2	3
5	K-MB-H5	10	40	H2-5	4	75	K-Ler1-3	2
6	K-SC-L2	10	41	W4-3	4	76	K-Rt-H5	2
7	K-Be-H3	10	42	K-Hf-L2	4	77	H2-1	2
8	CB3-1	8	43	K-Rt-H1	4	78	Ler 3-1	2
9	K-CK2-1	8	44	K-BG-H8	3	79	MB-H2	2
10	K-MB-H4	8	45	HF-L3	3	80	Hf-L7	2
11	K-Be-H1	7	46	K-BG-H1	3	81	PSV1-8*	2
12	Ler3-4	7	47	K-H4-1	3	82	PCV1-13*	2
13	K-CB1-1	7	48	K-NB1-1	3	83	Ler1-2	2
14	K-Ler3-1	6	49	K-Rt-H2	3	84	Ler2-1	2
15	K-Ab-H3	6	50	H4-5	3	85	K-Ler1-1	2
16	DR1-2	6	51	Hf-L4	3	86	K-W212 31	2
17	W2-4	6	52	K-BG-H2	3	87	K-NB1-2	2
18	Hf-L5	6	53	K-H4-5	3	88	Ler1-1	2
19	Hf-L6	6	54	K-O3-1	3	89	CB1-11	2
20	CB3-2	6	55	K-Rt-H3	3	90	H2-7	2
21	Ler3-3	6	56	Hf-L2-1 C	3	91	H2-2	2
22	K-CB2-6	6	57	K-Hf-L8	3	92	MB-H4	2
23	K-Ab-H2	6	58	K-Rt-H4	3	93	K-SV-H2	2
24	K-Ler2-2	6	59	PCB1-15*	3	94	DR3-4	1
25	K-Ler2-1	5	60	CB1-3	3	95	DR1-1	1
26	Hf-L1	5	61	DR4-4	3	96	NB2-1	1
27	NB4-3	5	62	MB-H5	3	97	MB-H3	1
28	K-Hf-H2	5	63	O1-2	3	98	W4-8	1
29	K-Hf-L9	5	64	W4-9	3	99	DR3-2	1
30	K-Ler2-3	5	65	PSV1-9*	3	100	W1-1	1
31	E3-3	5	66	W3-1	3	101	PCB2-2*	1
32	DR3-1	5	67	DR3-11	3	102	PCB3-3*	1
33	Ler4-1	5	68	W3-2	3	103	PCB3-4*	1
34	Hf-L2	5	69	K-BG-H3	3	104	DR3-5	1
35	K-Ler2-1	5	70	K-BG-H7	3	105	DR3-6	1

Continued

Serial No.	Isolate	Mean inhibition zone (mm)	Serial No.	Isolate	Mean inhibition zone (mm)	Serial No.	Isolate	Mean inhibition zone (mm)
106	W2-5	1	133	PCB1-4*	1	160	K-MB-L2	1
107	W4-4	1	134	PCB1-10*	1	161	K-NB3-1	1
108	DR3-3	1	135	DR3-7	1	162	K-O2-1	1
109	H3-6	1	136	DR4-2	1	163	K-Pe-L2	1
110	NB4-1	1	137	E3-2	1	164	K-CK3-1-1C	1
111	PE-L1	1	138	H2-4	1	165	K-CK3-7-1C	1
112	SC-L1	1	139	H3-2	1	166	Be-H2	1
113	W4-5	1	140	H4-7	1	167	CK3-7	1
114	K-SV-H3	1	141	O1-5	1	168	DR2-6	1
115	PK1-11*	1	142	W2-1	1	169	H3-1	1
116	PSV1-7*	1	143	PCB1-5*	1	170	H4-3	1
117	NB4-2	1	144	H2-3	1	171	MB-H3	1
118	PSV1-15*	1	145	K-CB4-1	1	172	MB-L3	1
119	PK1-12*	1	146	K-CK3-1	1	173	MS4-1	1
120	PK1-10*	1	147	K-CK3-2	1	174	Pe-L1	1
121	PCB1-13*	1	148	K-CK3-4	1	175	SV-H1	1
122	PK4-18*	1	149	K-CK3-5	1	176	H4-4	1
123	PK4-16*	1	150	K-CK3-6	1	177	H2-6	1
124	PCB1-6*	1	151	K-DR4-2	1	178	SC-L3	1
125	O1-3	1	152	K-Hf-L1	1	179	PE-H1	1
126	CB1-13	1	153	K-HF-L4	1	180	Ler2-4	1
127	H3-1	1	154	K-Hf-L5	1	181	NB3-2	1
128	MB-H1	1	155	K-Hf-L6	1	182	O2-2	1
129	PCB1-14*	1	156	K-Hf-L10	1	183	W211XX	1
130	W4-1	1	157	K-Ler1-2	1	184	W21XXX	1
131	PK4-15*	1	158	K-MB-H2	1			
132	PSV1-20*	1	159	K-MB-L1	1			

[†]The inhibition zone is the clearing zone in the interface between the tip of *A. euteiches* mycelia and the edges of the antagonistic bacterial colony. It is expressed in mm and each number is the average of six interaction zones that is rounded off to the nearest digit. * denote bacterial isolates from a previously existing bacteria culture collection.

Figure 3.7 Bacterial isolates exhibiting an antagonistic effect on *Aphanomyces euteiches* mycelia growth on potato dextrose agar, incubated at 23 °C for 5 d in the dark.

3.4.4 Zoospore germination inhibition assay

The antagonistic bacterial isolates that inhibited the mycelial stage of *A. euteiches* were able to reduce zoospore germination and subsequent growth to some extent when tested *in vitro* on PDA plate when applied as an undiluted stock dilution, and 47 isolates inhibited zoospore germination by as much as 75% or more relative to the control assay plates (Table 3.4). However, only 14 isolates maintained this level of control when applied as 100-fold dilution. Isolates such as DR1-3, Ler1-1 and W2-4 were among the isolates that exhibited the highest inhibitory effect towards zoospore germination (Table 3.4, Fig. 3.8). The presence of Trypticase soy broth used in growing the bacterial culture had no measurable effect on zoospore germination.

Table 3.4 *Aphanomyces euteiches* zoospore germination inhibition by antagonistic bacterial isolates *in vitro* assay.

S.N.	Bacterial isolates	Inhibition score†			
		Stock dilution (%)	Rank	100-fold dilution (%)	Rank
1	DR1-2	100	1	100	1
2	DR1-3	100	1	83	9
3	DR3-1	100	1	92	6
4	DR3-4	100	1	100	1
5	Ler1-1	100	1	100	1
6	Ler4-1	100	1	92	6
7	W2-4	100	1	92	6
8	K-Be-H3	100	1	67	15
9	K-CB1-1	100	1	33	34
10	K-CB2-6	100	1	100	1
11	K-Hf-L9	100	1	100	1
12	DR1-1	92	12	67	15
13	H2-1	92	12	50	27
14	Hf-L1	92	12	50	27
15	Hf-L2	92	12	75	11
16	Hf-L5	92	12	83	9
17	PK1-11*	83	17	17	50
18	PSV1-7*	83	17	75	11
19	PCB1-15*	83	17	75	11
20	CB1-3	83	17	17	50
21	CB3-1	83	17	58	20
22	H2-5	83	17	58	20
23	Hf-L7	83	17	58	20
24	Ler3-4	83	17	67	15
25	Ler4-2	83	17	75	11
26	CB1-11	75	26	17	50
27	DR4-4	75	26	33	34
28	E3-1	75	26	17	50
29	H2-7	75	26	8	64
30	H4-5	75	26	17	50
31	Hf-L4	75	26	25	43
32	Hf-L6	75	26	67	15
33	Ler 3-1	75	26	0	77
34	Ler3-3	75	26	17	50
35	MB-H5	75	26	33	34
36	NB2-1	75	26	0	77

Continued

S.N.	Bacterial isolates	Inhibition score			
		Stock dilution (%)	Rank	100-fold dilution (%)	Rank
37	NB4-2	75	26	58	20
38	NB4-3	75	26	58	20
39	O1-2	75	26	50	27
40	W4-3	75	26	33	34
41	W4-9	75	26	67	15
42	PSV1-15*	75	26	33	34
43	PSV1-8*	75	26	33	34
44	PK1-12*	75	26	17	50
45	PK1-10*	75	26	8	64
46	PCB1-13*	75	26	17	50
47	K-Hf-H2	75	26	0	77
48	K-CB2-4	67	48	50	27
49	K-CB2-2	67	48	50	27
50	K-CB2-3	67	48	58	20
51	K-CB2-1	67	48	50	27
52	K-MB-H5	67	48	50	27
53	K-Ler3-1	67	48	58	20
54	K-Ab-H2	67	48	33	34
55	K-Ler2-2	67	48	0	77
56	PSV1-9*	67	48	33	34
57	PCV1-13*	67	48	33	34
58	K-SC-L2	42	58	17	50
59	MB-H2	42	58	17	50
60	MB-H3	42	58	17	50
61	PK4-18*	42	58	8	64
62	PK4-16*	42	58	17	50
63	W4-8	42	58	25	43
64	PCB1-6*	33	64	0	77
65	DR3-2	33	64	25	43
66	H2-2	33	64	25	43
67	K-Be-H1	33	64	8	64
68	K-Ler2-1	33	64	25	43
69	Ler1-2	33	64	25	43
70	O1-3	33	64	25	43
71	K-Ab-H3	33	64	0	77
72	CB1-13	25	72	8	64
73	H3-1	25	72	17	50
74	Ler2-1	25	72	8	64

Continued

S.N.	Bacterial isolates	Inhibition score			
		Stock dilution	Rank	100-fold dilution	Rank
75	MB-H1	25	72	8	64
76	MB-H4	25	72	0	77
77	W1-1	25	72	8	64
78	W3-1	25	72	17	50
79	PCB1-14*	25	72	8	64
80	PCB2-2*	17	80	0	77
81	PCB3-3*	17	80	0	77
82	PCB3-4*	17	80	0	77
83	DR3-5	17	80	0	77
84	DR3-6	17	80	0	77
85	DR3-11	17	80	0	77
86	HF-L3	17	80	0	77
87	W2-5	17	80	0	77
88	W3-2	17	80	0	77
89	W4-1	17	80	0	77
90	W4-4	17	80	0	77
91	PK4-15*	17	80	8	64
92	PSV1-20*	8	92	8	64
93	PCB1-4*	8	92	0	77
94	PCB1-10*	8	92	0	77
95	DR3-3	8	92	0	77
96	DR3-7	8	92	0	77
97	DR4-2	8	92	0	77
98	E3-2	8	92	0	77
99	H2-4	8	92	0	77
100	H3-2	8	92	8	64
101	H3-6	8	92	0	77
102	H4-7	8	92	0	77
103	NB4-1	8	92	8	64
104	O1-5	8	92	0	77
105	PE-L1	8	92	0	77
106	SC-L1	8	92	0	77
107	W2-1	8	92	0	77
108	W4-5	8	92	0	77
109	PCB1-5*	0	109	0	77
110	H2-3	0	109	0	77
111	K-Ler2-3	0	109	0	77

[†]*Aphanomyces euteiches* zoospore germination inhibition was determined using a 0-3 scale. Scores are a total of eight observations (i.e., microscopy fields) from two assay plates for each dilution. * denote bacterial isolates from a previously existing bacteria culture collection.

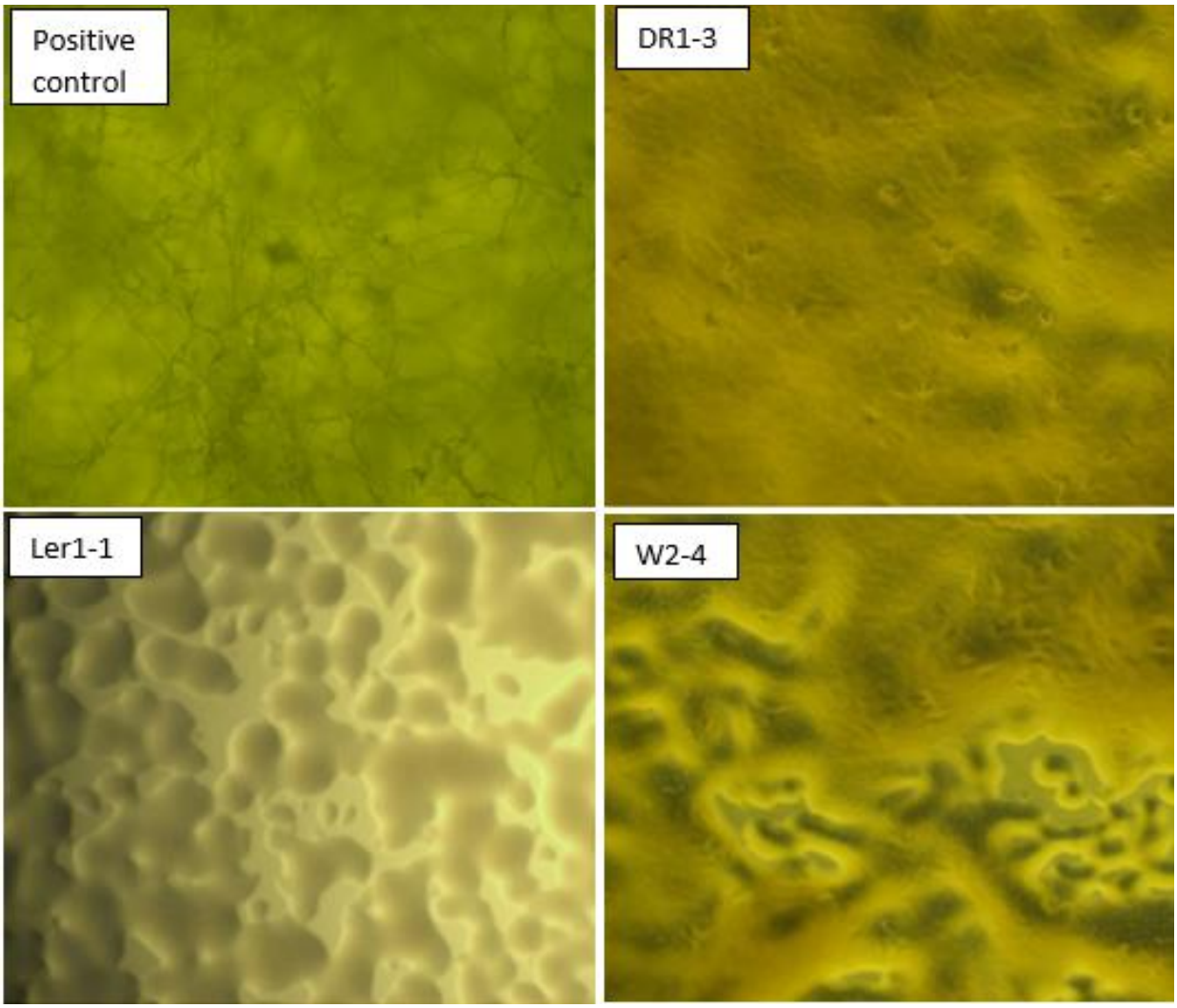

Figure 3.8 Complete *A. euteiches* zoospore germination inhibition by antagonistic bacteria (DR1-3, Ler1-1 and W2-4). Evaluation of *A. euteiches* zoospore germination inhibition was determined using a 0 (no germination) to 3 (heavy germination) scale, based on hyphal growth. Scores are a total of eight observations (i.e., microscopy fields) from two assay plates for each bacterial culture dilution. The positive controls were PDA assay plates consisting of *Aphanomyces euteiches* zoospore challenged with autoclaved distilled water. The assay plates were incubated at 23 ℃ for 5 d under dark conditions.

Pearson's correlation analysis (Table 3.5) was highly significant ($p \leq 0.001$) although the correlation between the isolate antagonistic potential towards *A. euteiches* mycelia growth generally were not strong. The correlation with zoospore germination was relatively strong ($r = 0.78$) and significant ($p \leq 0.001$).

Table 3.5. Pearson correlation coefficient (r) analysis between mycelia and zoospore inhibition at stock and 100-fold dilution of the antagonistic bacterial isolates.

	Mycelia inhibition	Zoospore germination inhibition†	
		Stock dilution	100-fold dilution
Mycelia inhibition	1	0.52***	0.48***
Stock dilution		1	0.78***
100-fold dilution			1

† Correlation analysis was performed to determine whether mycelia inhibition and zoospore germination inhibition at stock concentration and 100-fold dilution of the antagonistic bacteria were quantitatively related. Thus, Pearson correlation coefficient (r) was performed using SAS computer package. Pearson Correlation Coefficients, Prob > |r| under H0: Rho=0. *** denote p values ≤ 0.001.

3.4.5 Identification of the antagonistic bacterial isolates

Based on the 16S rDNA gene characterization, the antagonistic bacteria fall into 18 different genera. Genus-level grouping revealed that *Bacillus* sp., *Pseudomonas* spp., *Paenibacillus sp.*, *Lysobacter sp*. and *Streptomyces sp.* were the top five dominant groups (Fig. 3.9). More specifically, the top 31 bacterial species exhibiting the highest antagonistic effect towards *A. euteiches* mycelia are presented in Figure 3.10.

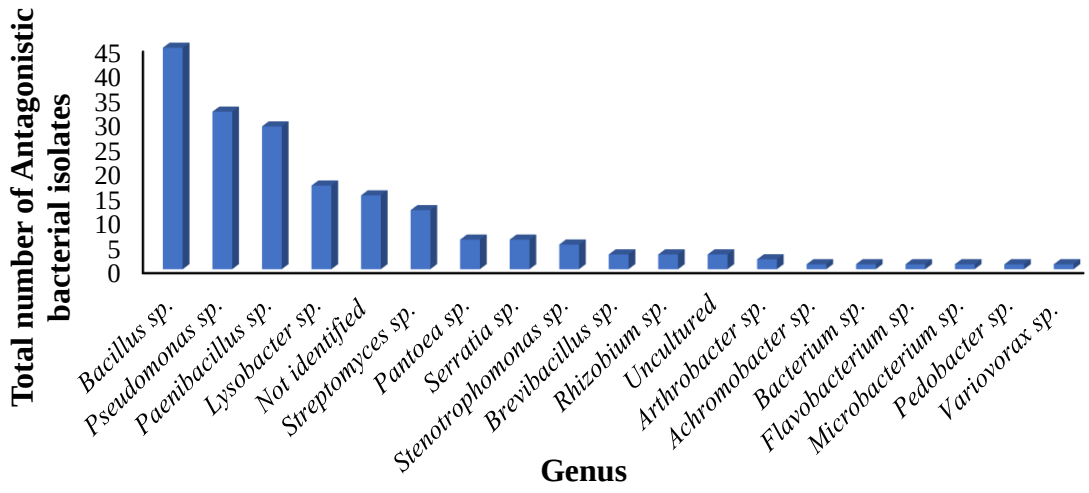

Figure 3.9 Genus-level grouping of bacterial isolates exhibiting antagonistic effects towards *Aphanomyces euteiches* mycelia.

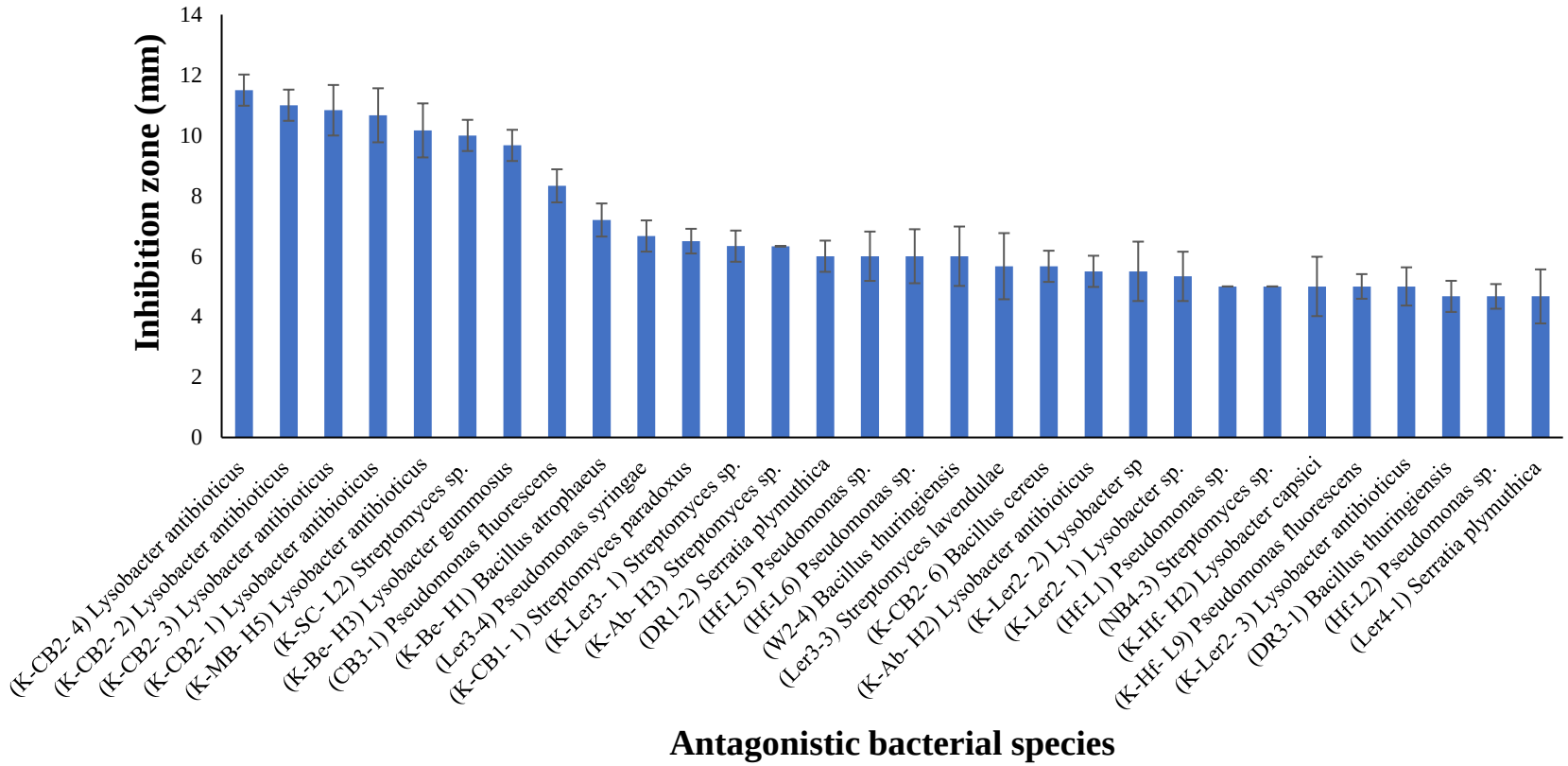

Figure 3.10 Bacterial species exhibiting antagonistic effects towards *A. euteiches* mycelial growth. These antagonistic bacterial species were the top 31 isolates that possessed the highest inhibition potential towards mycelia growth. The inhibition zone for these isolates ranged from 5 mm to 12 mm. Error bars indicate standard deviations. The letters and numbers found in the parentheses before the species name indicate the code for the respective isolates.

From a total of 47 isolates that inhibited zoospore germination, reference strains of three isolates were found to be Level Two organisms according to the Global Bioresource Center generally known by ATCC. Thus, these isolates were excluded from further study in this project. Although three isolates, namely K-CB2-4 (*Lysobacter antibioticus*), PCV1-13 (*Rhizobium lemnae*) and PSV1-9 (*Rhizobium lemnae*) showed less than 75% zoospore germination inhibition, they were included for further study. The reasons for their inclusion were because isolate K-CB2-4 (*Lysobacter antibioticus*) showed the highest mycelial growth inhibition (12 mm) and the other two isolates were *Rhizobium* species. *Rhizobium* species form an endosymbiotic nitrogen-fixing association with roots of legumes and thus a biocontrol agent derived from this group of bacteria would provide additional benefits apart from controlling aphanomyces root rot in field pea. Results indicated that zoospore germination inhibition potential varied among isolates when applied as a stock solution and 100-fold dilutions (Fig. 3.11). Moreover, greater number of encysted circular *A. euteiches* zoospores were observed at stock solution than 100-fold dilutions.

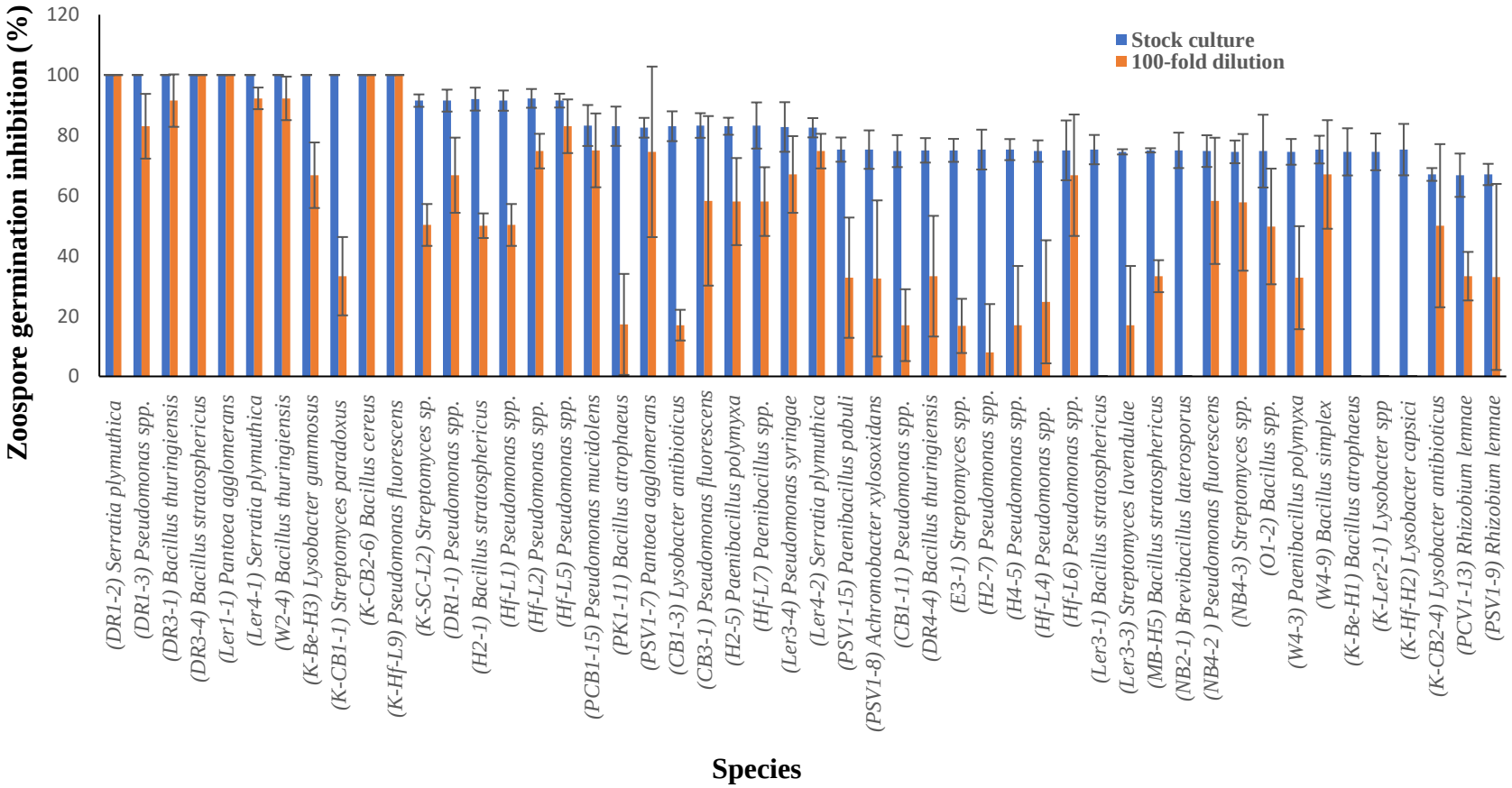

Figure 3.11 Bacterial species exhibiting 75% or more antagonistic effect towards *A. euteiches* zoospore germination (i.e. compared to a control plate). These antagonistic bacterial species are the top 47 isolates that possessed the highest inhibition potential towards zoospore germination. Although three isolates, namely K-CB2-4 (*Lysobacter antibioticus*), PCV1-13 (*Rhizobium lemnae*) and PSV1-9 (*Rhizobium lemnae*) showed less than 75% zoospore germination inhibition, these isolates were included for further study in this project. Error bars indicate standard deviations. The letters and numbers found in the parentheses before the species name indicate the code for the respective isolates.

3.5 DISCUSSION

3.5.1 Relationship between total culturable heterotrophic bacteria and soil characteristics

Pearson's correlation analysis indicated a significant (p = 0.02) positive correlation between total heterotrophic bacteria and SOC. However, significant correlations were not detected between pH and SOC, or pH and total heterotrophic bacterial count. The significant positive correlation between SOC and total heterotrophic bacteria could be because soil bacteria typically are favored by a readily available carbon source that is required for survival and fostering the microbial community (Souza et al., 2015) and this finding was consistent with the findings of Tripathi et al. (2013).

Soil bacteria are one of the important components of microbial communities which play a role in most nutrient transformations particularly by regulating the dynamics of soil organic matter, soil carbon sequestration, modifying soil physicochemical structure and function, and enhancing the efficiency of nutrient acquisition by plants and promoting plant growth and development (Singh et al., 2011). Heterotrophic bacteria as a major component of soil bacteria also participate in each of these activities. The present study on total heterotrophic bacterial population identified a wide range of variation across the sampling locations with a minimum (5.1×10^6 CFU g^{-1}) at Humboldt and maximum (7.7×10^8 CFU g^{-1}) at Mossbank (Table 3.1). Such variation in the total heterotrophic bacteria across the sampling locations could be associated with the difference in cropping history and agronomic management practices as reported by Hartmann et al. (2006) and Figuerola et al. (2012). Moreover, a report by Bais et al. (2004) indicated that such variation could be due to the complex positive and negative interactions in the rhizosphere region which may include the release of fatty acid, protein, amino acids, antimicrobial compounds and the presence or absence of suitable carbon sources for bacterial cell growth and reproduction.

Soil pH is another important parameter that affects soil abiotic factors such as nutrient availability and solubility of metals, and it may also control biotic factors, such as the biomass composition of fungi and bacteria (Rousk et al., 2009). The pH of the soil samples used in this study ranged from 5.0 to 8.5 (Table 3.1). This result was consistent with a report by the Canola Council of Canada (2017) which states that although most cultivated soils in western Canada are alkaline or neutral, large areas of soil with a pH of 6.0 or less occur in Saskatchewan, Alberta, northeast British Columbia and Ontario.

Soil organic carbon determines soil quality by affecting the chemical, physical and biological functions taking place in soils systems, and it affects soil properties such as moisture holding capacity, nutrient availability, diversity and activity of soil organisms (Schjønning et al., 2018). The SOC contents of the samples used in this study ranged from a minimum of 8.8 mg g^{-1} at Edam to a maximum of 52.2 mg g^{-1} at Goodsoil in a low spot (Table 3.1).

3.5.2 Primary screening of antagonistic bacteria

The crowded plate assay technique was selected as a primary screening method to isolate high numbers of bacteria possessing biocontrol activity against the mycelial growth stage of *A. euteiches*. From a total of 43 rhizosphere soil samples collected from commercial field pea fields across Saskatchewan, 184 bacterial isolates antagonistic to *A. euteiches* mycelia were identified, of which 22 were from a previously existing bacterial culture collection (Table 3.3), and these isolates were grouped into 18 different genera (Fig. 3.9). The proportion of antagonistic bacteria to total culturable heterotrophic bacteria ranged from 0 to 4 % across the sampling locations.

The identification of antagonistic bacteria from an existing culture collection of roots associated rhizobacteria is an indication that biocontrol activity towards *A. euteiches* is not limited to bacteria isolated from infested soils but seems to be a general phenomenon found in a variety

of soils and/or plant-associated bacteria. Similarly, Hanson and Fernandez (2002) identified bacterial isolates antagonistic to *Fusarium graminearum* and other cereal pathogens from an existing culture collection and new isolates from soil and crop residues.

The diversity of bacterial isolates possessing antagonistic activity towards *A. euteiches* could be an indication of the high degree of competition in the rhizosphere region. Although soil microbial communities are highly diverse and ubiquitous in nature, their distribution is not uniform in various habitats due to variations in factors such as soil moisture, organic and inorganic chemicals, soil organic matter and type of vegetation and its growth stages (Prashar et al., 2014; Paul, 2014).

Generally, the majority of soil bacteria are concentrated in the rhizosphere region where nutrients are constantly released as root exudates (Jia et al., 2015). Root exudates are mediators of plant-microbe interactions and microbes interact via chemotactic responses leading to root colonization (Prashar et al., 2014). Therefore, microbes have specialized strategies such as competitive antagonism for survival and fostering community development in this region (Montesinos, 2003). Competitive antagonistic mechanisms have the potential to directly inhibit pathogen growth and degrade virulence factors or pathogen cell-wall components (Maheshwari, 2017). Such antagonistic mechanisms include the production of antibiotics, toxins, hydrogen cyanide (HCN) and hydrolytic enzymes (chitinases, proteases, lipases) (Pereg and McMillan, 2015). Rhizosphere bacteria are adapted to live in proximity with the host plant roots. Consequently, bacteria from this region possessing antagonistic effects towards root pathogens are promising candidates for biocontrol agent development.

3.5.3 Mycelia growth inhibition assay

The crowded plate assay technique was an essential tool for screening bacterial isolates possessing biocontrol against *A. euteiches* under laboratory conditions. However, this technique was not well suited for comparative analysis of the antagonistic potential among isolates as multiple isolates may be involved in the biocontrol activity observed on the assay plates. As a result, a dual plate assay technique was used. This technique allowed for the measurement of the antagonistic potential by assessing the degree to which an inhibition zone developed at the interface between the tip of *A. euteiches* mycelia and isolate colony edge.

The antagonistic bacterial isolates inhibited *A. euteiches* mycelial growth to varying degrees, ranging from a minimum of 1 mm to a maximum of 12 mm zone of inhibition (Table 3.3). Isolates such as K-CB2-4 (*Lysobacter antibioticus*), K-SC-L2 (*Streptomyces sp*.), CB3-1 (*Pseudomonas fluorescens*), K-Be-H1 (*Bacillus atrophaeus*) and DR1-2 (*Serratia plymuthica*) produced the highest biocontrol activity with an inhibition zone of 6 mm or more. Moreover, microscopic observation of the *A. euteiches* mycelia tips around the inhibition zone revealed altered mycelia morphology, branching size and curling of hyphal tips suggesting that mycelia growth was inhibited by direct effect of inhibitory compounds released from the isolates. Although *A. euteiches* has two stages of mycelia growth (i.e., pre- and post-infection), the main stage that causes disruption of root tissue and collapse of the root system occurs during the post infection phase (Hughes and Grau, 2013). Therefore, isolates that inhibit mycelia growth using *in vitro* assay may have the capacity to reduce the infective potential of *A. euteiches*.

Isolates such as K-CB2-4 (*Lysobacter antibioticus*), K-Hf-H2 (*Lysobacter capsici*) and other members of the genus *Lysobacter* are gram-negative bacteria widely distributed in diverse ecosystems, including soil, rhizosphere, and freshwater habitats and they are known for their

gliding motility and the ability to lyse other microorganisms including fungi (Reichenbach, 2006; Islam, 2008). Hence, *A. euteiches* mycelia growth inhibition by *Lysobacter* species could be due to the production of lytic enzymes and other secondary metabolites that have biocontrol effects. A similar study by Ko et al. (2009) indicated that *Lysobacter antibioticus* HS124 isolated from rhizosphere soil produced lytic enzymes such as beta-1,3-glucanase, chitinase, lipase, protease and an antibiotic compound that was identified as 4-hydroxyphenylacetic acid through various chromatography techniques, had anti-fungal activity against *Phytophthora capsici*, a destructive pathogen of pepper plants. Another report by Islam et al. (2004) indicated that *Lysobacter sp*. SB-K88 inhibited mycelia growth of *Aphanomyces cochlioides* Drechsler by producing a metabolite known as xanthobaccin-A. Eighteen different strains of *Lysobacter* spp. isolated from soils suppressive to *Rhizoctonia solani* showed strong *in vitro* activity against *Rhizoctonia solani*, *Pythium ultimum, Aspergillus niger*, *Fusarium oxysporum*, and *Xanthomonas campestris* (Gómez et al., 2015).

Isolate K-SC-L2 (*Streptomyces sp*.) inhibited mycelia growth of *A. euteiches* with an 8 mm zone of inhibition and this was found to be the maximum zone of inhibition recorded among the isolates belonging to the genus *Streptomyces* isolated in this study (Table 3.3). *Streptomyces* produce antibiotics and volatile organic compounds that are active against various plant pathogens including fungi (de Lima et al., 2012). These bioactive secondary metabolites are often species-specific and allow them to develop symbiotic interactions with plants by protecting them from various pathogens (Vurukonda et al., 2018). Therefore, *A. euteiches* mycelia growth inhibition by isolates from this genus could be due to the antagonistic effect of volatile organic compounds and antibiotics released against the extending *A. euteiches* mycelia observed *in vitro*. Similarly, laboratory studies have shown that *Streptomyces* exhibit inhibitory activity against *Magnaporthe*

oryzae, which is the causative agent for a rice blast (Law et al., 2017). A report by Ohike et al. (2017) indicated that *Streptomyces* species were able to suppress growth of eight different pathogenic fungi including *Rhizoctonia solani* on a dual plate assay. Although *Streptomyces* are rhizosphere soil organisms, they also are efficient colonizers of plant tissues that extends from root to aerial parts (Vurukonda et al., 2018). Moreover, they are important sources of many clinically useful antibiotics of natural origin such as neomycin and chloramphenicol (Sharma et al., 2014).

Isolates belonging to the genus *Pseudomonas* also were shown to have biocontrol activity against *A. euteiches* mycelia growth with a maximum inhibition zone of 8 mm observed for isolates CB3-1 (*Pseudomonas fluorescens*). *Pseudomonas fluorescens* is known to possess plant growth promoting characteristics and strains of this species are well known for their role in plant growth promotion, induced systemic resistance, biological control of pathogens (Ganeshan et al., 2005) and have important traits in bacterial fitness including the ability to adhere to soil particles and to the rhizoplane, motility and prototrophy (i.e., to synthesize all of the required growth factors but supplying these to cultures resulting in a faster growth rate) (Panpatte et al., 2016). Wang et al. (2003) reported that strains of *Pseudomonas fluorescens* exhibited antagonistic effects against *Pythium ultimum, Rhizoctonia solani, Fusarium avenaceum* and *Ascochyta pisi* of field pea *in vitro*.

Isolate K-Be-H1 (*Bacillus atrophaeus*), W2-5 (*Bacillus cereus*) and other members of the genus *Bacillus* isolated in this study are characterized by the formation of dormant endospores when challenged with unfavorable growth conditions (Zeigler and Perkins, 2015). Among the isolates identified as *Bacillus* in this study, isolate K-Be-H1 (*Bacillus atrophaeus*) produced a maximum zone of inhibition of 7 mm. *Bacillus* spp. produce several kinds of bioactive secondary metabolites such as bacillomycin, fengycin, mycosubtilin and zwittermicin which are effective in

controlling various plant pathogens (Pal and McSpadden, 2006). A study report by Wakelin et al. (2002) indicated that *Bacillus* spp. such as *Bacillus mycoides* MW 27, *Bacillus cereus* 15'80 and *Bacillus subtilis* PT 69 exhibited biocontrol activity against *A. euteiches* mycelia when evaluated *in vitro*. Similarly, Banerjee et al. (2018) reported that *B. cereus* UW85 suppressed damping off disease on alfalfa, a disease caused by *Phytophthora megasperma* f. sp. *medicaginis* . Moreover, an earlier study by Lozano et al. (2016) reported that *B. cereus* UW85 produces two antibiotics, namely zwittermicin-A and kanosamine which exhibit broad-spectrum biocontrol effects that contribute to the suppression of alfalfa seedling damping off.

Some strains of the *Serratia* species have been reported to have antifungal properties and are root colonizers (Scher et al., 1988; Grimont and Grimont, 1992; Wang et al., 2003); however, they are not always beneficial to plants (Weissmann and Gerhardson, 2001). In the present study, isolate DR1-2 (*Serratia plymuthica*) exhibited the highest antagonistic effect towards mycelia growth with a maximum zone of inhibition of 6 mm among the isolate identified as *Serratia* spp. In a study conducted to evaluate *S. plymuthica* strain HRO-C48 for biocontrol of the fungal pathogens *Verticillium dahliae* and *Phytophthora cactorum* in greenhouse trials, bacterial inoculation reduced the percentage of Verticillium wilt and Phytophthora root rot in pea plants (Kurze et al., 2001).

Although bacterial species belonging to the genus *Bacillus*, *Pseudomonas* and *Streptomyces* are widely used as biocontrol agents (Banerjee et al., 2018), other isolates belonging to the genus *Rhizobium*, *Paenibacillus*, *Pantoea*, *Stenotrophomonas*, *Brevibacillus*, *Arthrobacter*, *Achromobacter*, *Bacterium*, *Flavobacterium*, *Microbacterium*, *Pedobacter* and *Variovorax* were also identified as having biocontrol effects against mycelia growth with inhibition zones ranging from a minimum of 1 mm to a maximum of 5 mm (Table 3.3) suggesting that *A. euteiches* mycelia

growth inhibitory bioactive molecules may be shared among various bacterial species belonging to different genera, and/or distinct bioactive molecules from these antagonistic bacteria might have been involved in the biocontrol interactions observed *in vitro* assay in this study. As a result, *in vitro* inhibition of the *A. euteiches* mycelia stage holds great promise for the development of biocontrol agents that may have the capacity to reduce the infective potential of *A. euteiches*.

3.5.4 Zoospore germination inhibition assay

Inhibition of *Aphanomyces euteiches* mycelia was an important assay to identify bacteria possessing mycelia growth inhibitory potential from a large background of soil bacteria. Subsequently, there was a need to conduct zoospore germination inhibition assays as it was believed that isolates which are suppressive to both infective stages (mycelia and zoospores) could be more suppressive to *A. euteiches*, and screening of these isolates for biocontrol potential *in vivo* may provide a greater biocontrol effects than isolates that inhibit a single phase of the pathogen.

The zoospore germination inhibition assay was conducted using a stock culture and a 100-fold dilution of each isolates under identical conditions for 24 h for each isolate. These assays evaluated whether an isolate could inhibit zoospore germination and whether the inhibition was influenced by the concentration of the bacterial culture. The method used was consistent with the one used by Wakelin et al. (2002) to evaluate biological control of aphanomyces root rot of pea with spore-forming bacteria and was not intended to provide specific dose response information. However, even given its limitations, the results of this assay contributed to detecting candidate biological control agents that have biocontrol effects towards the zoospore germination stage.

The zoospore germination inhibition potential of the antagonistic bacterial candidate biocontrol isolates evaluated in this study ranged from 0 to 100 % when applied as a stock culture and at 100-fold dilutions (Table 3.4). The number of isolates that inhibited zoospore germination

by 75% or more when applied as a stock dilution dropped to 28% when applied as 100-fold dilution suggesting that the biocontrol effects towards zoospore germination of some isolates dependent on cell concentrations. This was consistent with the findings of Heungens et al. (2001). Heungens et al. (2001) observed that *Bacillus cepacia* AMMDR1 significantly reduced aphanomyces root rot in pea only when the bacteria were applied at high population densities at the site of zoospore inoculation. Moreover, isolates such as DR1-2 (*Serratia plymuthica*), DR3-4 (*Bacillus stratosphericus*), K-CB2-6 (*Bacillus cereus*) Ler1-1 (*Pantoea agglomerans*), and K-Hf-L9 (*Pseudomonas fluorescens*) were among the isolates that exhibited complete inhibition of zoospore germination when applied as both a stock and 100-fold dilutions.

These potential candidate antagonistic bacteria have been used as a biocontrol agents against various plant diseases by others (Ganeshan and Manoj, 2005; Srividya et al., 2012; Castillo et al., 2013; Hong et al., 2016) and in most cases the mechanism of biocontrol activity was through the release of secondary bioactive compounds such as antibiotics and cell wall degrading enzymes (Wright et al., 2001; Ganeshan et al., 2005; Liu et al., 2010; Castillo et al., 2013). The identification of isolates inhibitory to both infective stages (mycelia and zoospore) holds great promise for effective biological control of aphanomyces root *in vivo*. Moreover, these bacteria were isolated from Saskatchewan fields, and thus should be adapted to regional growing conditions.

3.6 CONCLUSION

The present study consisted of isolation and identification of bacterial isolates antagonistic to *A. euteiches* mycelia and zoospore growth stages under laboratory conditions. In conclusion, evidence is presented in this study that the lifecycle of *A. euteiches* can be interrupted using rhizosphere bacteria and the results were consistent with prior findings by Heungens et al. (2001), Wakelin et al. (2002), and Islam et al. (2004). Isolates vary in antagonistic potential towards both mycelia and zoospore stages of the pathogen. Some isolates which were inhibitory to mycelia stages of *A. euteiches* did not inhibit zoospore germination.

Molecular analysis determined that the antagonistic bacterial isolates were from 18 different genera. Variations of inhibition potential and diverse identity among isolates suggests that the mechanisms by which biocontrol was achieved such as the production and secretion of inhibitory compounds and/or the mode of action exerted by the inhibitory metabolites likely varies among isolates.

Identification of isolates inhibitory to *A. euteiches* growth stages holds great promise for the development of microbial biocontrol agents. Irrespective of the inhibition level and the nature of the anti-pathogen interaction observed *in vitro*, control of aphanomyces root rot and efficacy of the antagonist will only be observed after successful establishment and survival in the root zone of the field pea. Therefore, there is a need to conduct further studies aimed at assessing the efficacy of the promising isolates *in vivo* experiments involving a host plant.

4. ASSESSMENT OF BIOCONTROL BACTERIA IN GROWTH CHAMBER TRIALS USING FIELD PEA AS A TEST CROP

4.1 ABSTRACT

Aphanomyces root rot caused by *A. euteiches* is one of the most destructive root diseases of the field pea and other leguminous plant species in different parts of the world. Effective control methods are not available in Canada. Therefore, the aim of this study was to assess the potential for biological control of aphanomyces root rot in field pea grown in sterile vermiculite and non-sterile field soil using antagonistic bacterial isolates identified earlier in this project (chapter 3).

Growth chamber experiments were conducted using 47 bacteria that inhibited *A. euteiches* mycelia growth and zoospore germination in *in vitro* assays. Isolates such as PCV1-13 (*Rhizobium lemnae*), DR1-2 (*Serratia plymuthica*), DR1-3 (*Pseudomonas* spp.), DR4-4 (*Bacillus thuringiensis*), H2-1 (*B. stratosphericus*), H2-5 (*Paenibacillus polymyxa*), H4-5 (*Pseudomonas* spp.), Ler3-1 (*B. stratosphericus*) and MB-H (*B. stratosphericus*) completely inhibited aphanomyces root rot in field pea grown in vermiculite. Isolates K-Hf-H2 (*Lysobacter capsici*) and K-CB2-6 (*B. cereus*) appeared to exacerbate disease development when vermiculite was used as a growth medium. Compared to the biocontrol assessment in vermiculite the level of aphanomyces root rot development was much higher when non-sterile field soil was used as a growing medium and all treatments had some level of aphanomyces root rot. Isolates K-Hf-L9 (*Pseudomonas fluorescens*), PSV1-7 (*Pantoea agglomerans*) and K-Hf-H2 (*L. capsici*) were identified as having the highest biocontrol effects when non-sterile field soil was used as a growing medium. Therefore, these promising biocontrol agents can be considered for future studies aimed at evaluating biocontrol efficacy against aphanomyces root rot in field pea in field conditions in Saskatchewan.

4.2 INTRODUCTION

Development of biological control agents against aphanomyces root rot in pea offers an alternative option for growers in addition to the traditional and chemical methods that exist today. Several studies have used *in vitro* screening of large numbers of microorganisms against plant pathogens as an initial step in the process of finding biological control agents. However, current *in vitro* screening techniques, such as dual-plate assays, ignore the influence of biotic and abiotic factors that exist in the rhizosphere soil (González et al., 2010). Therefore, *in vitro* assays provide limited information because the procedures typically exclude the factors involved in disease causation such as the host, pathogen and environment which form the disease triangle (Francl, 2001). A candidate bacterial isolate is considered to be a true biocontrol agent against the soil-borne root pathogen *A. euteiches* when it possesses key features such as compatibility with the pea roots and potential to colonize and survive in the rhizosphere soil (Weller, 2007).

Irrespective of the inhibition level and the nature of the anti-pathogen interaction observed *in vitro*, control of root rot diseases and efficacy of the antagonist will only be observed after successful establishment and survival in the root zone of the host plant (John, 2001). Therefore, there is a need to conduct further *in vivo* experiments involving a host plant. However, in most cases, field experiments are impracticable for screening methods right after *in vitro* assays because isolates identified *in vitro* may include strains which may cause phytotoxicity and phytopathogenicity to the host plant itself, either as a primary or a secondary pathogen (Walton, 1996). Therefore, an intermediate *in vivo* assay such as growth chamber studies which have the capacity to evaluate biocontrol potential and screen isolates with desirable attributes for biocontrol development should be conducted.

Consequently, this chapter describes the development of a plant-based growth chamber experiments using sterile vermiculite and subsequently non-sterile agricultural soil for the assessment of potential biocontrol of aphanomyces root rot of field pea. The objectives were: 1) to determine the efficacy of the antagonistic bacteria to suppress aphanomyces root rot of pea disease development both using vermiculite and agricultural soil; and 2) identify the antagonistic bacteria with desirable properties for biocontrol development.

4.3 MATERIAL AND METHODS

4.3.1 Growth conditions and experimental design

A series of experiments were carried out in the controlled environment facility at the College of Agriculture and Bioresources at the University of Saskatchewan, Canada using field pea (*Pisum sativum* L) as the test crop grown in two different media. These experiments assessed the efficacy of previously identified bacteria possessing biocontrol properties against *A. eutieches in vivo* as biocontrol agents when used as an inoculant. The first growth medium was vermiculite (SUNGRO HORTICULTURE, USA) which has been successfully used in aphanomyces root rot assays by Dr. Sabine Banniza (University of Saskatchewan Plant Science-Crop Development Centre). This soil-less medium enables water retention and rapid separation of roots with minimal damage and discoloration to the root system. The second medium used for this study was non-sterile agricultural field soil. The soil was a Brown Chernozem collected near Central Butte from the top 15 cm of an Ardill association soil (Baan et al., 2009) and has a pH of 7.8 and 9.8 mg g^{-1} soil organic carbon.

All growth chamber experiments were conducted using a completely randomized design with four replicates. A series of trials were conducted to accommodate the number of biocontrol isolates (n=47) assessed. A total of five trials were conducted using vermiculite, with each trial evaluating up to 10 biocontrol isolates. Following the experiments using vermiculite as a growth medium, two trials were conducted using the field soil, with each trial assessing 10 bacteria. The growth chamber room temperature was set to 22 °C day/21 °C night with a day length of 16 h. Light intensity ranged from 300 to 390 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The light bulbs in the room were Phillips T-5 Fluorescence bulb # 835 (ON, Canada). Plant positions were re-randomized at each watering

period throughout the experiment to minimize the impact of variations in light, temperature and humidity. The entire growth chamber experiment was executed in accordance with the University of Saskatchewan biosafety permit regulations. Thus, only isolates with Biosafety Level 1 status were tested.

4.3.2 Water holding capacity determination

Water holding capacity was determined according to Rowell (1994). Throughout the experiment, the moisture level for each pot was maintained at 80% of the water holding capacity by additions of sterile water.

4.3.3 Biocontrol assessment in vermiculite

Pea (cv. CDC Meadow) seeds were obtained from the Crop Development Centre (courtesy Dr. Tom Warkentin), University of Saskatchewan. The pea seeds were surface sterilized by soaking in ethanol (65% v/v) for 3 min and sodium hypochlorite (1.2% v/v) for 5 min, followed by 10 rinses in sterile tap water (Vincent, 1970). Four surface sterilized pea seeds were sown at equal depth (approximately 2.5 cm below the vermiculites surface) in 2500 mL pots (Fig. 4.1) containing 260 g vermiculite and covered with aluminum foil until germination. Immediately after emergence, the pea plants were thinned to two plants per pot.

A total of 47 antagonistic bacterial isolates which inhibited mycelia growth and zoospore germination by 75 % or more in the previous study in this project were taken from -80 °C storage and cultured on 1/10 TSA plates for 3 d at 28 °C. A single bacterial colony was then scraped from the surface of agar plates into 300 mL half strength TSB. The bacterial isolates were cultured on a rotary shaker that was adjusted at 120 rpm at 28 °C for 48 h. The cell growth was measured in

terms of optical density (OD_{660}) using a Thermo Scientific Evolution 60S UV-Visible Spectrophotometer (Thermo Scientific, USA).

Figure 4.1 Growth chamber trial setup. Pea seeds are planted into 2500 mL pots containing vermiculite. Water holding capacity was maintained at 80%.

Bacterial suspensions were concentrated by centrifugation (15 min at 5000 x g), washed three times in 150 mL sterile phosphate buffered saline (PBS) solution, and resuspended in sterile tap water. The bacterial cell density in the suspension was obtained as an absorbance value and converted into CFU mL^{-1}. Finally, the inoculum volume was adjusted to a target concentration of $1x10^8$ CFU mL^{-1} as described in Jones et al. (2009).

To allow pre-colonization of pea root, 6 d after germination, each pea plant was inoculated with 5 mL of one of the bacterial suspensions containing $1x10^8$ CFU mL^{-1} per plant. Three days after bacterial inoculation, and typically 10 d after seed germination, 5 mL of *A. euteiches* zoospore suspension (Section 3.4.4) was inoculated to deliver 0.5 x 10^4 zoospore mL^{-1}. Inoculants were added by pipetting the solutions into the root zone immediately beside the point of plant emergence. The following test controls were: 1) Control A: Pea plants inoculated with 5 mL of

antagonistic bacterial suspension at $1x10^8$ CFU mL^{-1} per plant only; 2) Control B: Pea plants inoculated with 5 mL of *A. euteiches* zoospore suspension at 0.5 x 10^4 zoospore mL^{-1} per plant only; and 3) Control C: Uninoculated pea plants.

4.3.4 Data collection and analysis

Plants were grown for 28 d at which time they were removed from each pot and assessed for the level of disease development. Initially, the roots were washed, and adhering vermiculite was removed. The level of disease development was scored and recorded using a 0 to 4 scale (Fig. 4.2) as described in Wakelin et al. (2002): 0 = No symptoms; roots healthy and white; 1 = Initial symptoms of root rot; discoloration, usually a light tan color, in sections of the root system; 2 = Discoloration of most or all the root system, usually still of a tan color. Small watery lesions may be present on the root and around the hypocotyl/epicotyl regions; 3 = Advanced disease symptoms. Dwarfing of the plant and yellowing of the lower leaves. Extensive darkening and discoloration of the root system and extensive lesion formation; 4 = Root entirely rotted / plant dead.

Analysis of variance (ANOVA) was used to determine the biocontrol activity of the isolates of each set. Moreover, multiple comparisons were made between the controls and each treatment using Statistical Analysis System (SAS version 9.3).

Figure 4.2 Aphanomyces root rot disease score rating scale in pea (Wakelin et al., 2002).

4.3.5 Biocontrol assessment in non-sterile soil.

Isolates exhibiting biocontrol effects with a disease score for aphanomyces root rot up to 1 when vermiculite was used as a growing medium were further assessed for biocontrol assessment in non-sterile soil. These subsequent growth chamber trials were set up in a manner similar to that described in Section 4.3.3. However, the number of antagonistic bacterial isolates to be tested was reduced from 47 to 20, and vermiculite was replaced with 500 g field soil (Fig. 4.3).

Initially, non-sterile airdry field soil was passed through a 2 mm mesh sieve and thoroughly mixed by hand. Five hundred grams of soil was placed in 500 mL capacity pots and the moisture content was adjusted to 80% water holding capacity as described in Section 4.3.2. Each pot was covered with aluminum foil to maintain moisture and incubated for 10 d on a growth chamber bench before sowing peas. The moisture level was continuously monitored and adjusted to 80% water holding capacity every other day. Four surface sterilized pea (CDC Meadow) seeds were placed approximately 2.5 cm below the soil surface in all pots and covered with aluminum foil until germination. Immediately after emergence, the pea plants were thinned to two plants per pot.

The top 20 antagonistic bacterial isolates which inhibited aphanomyces root rot development in the previous study which used vermiculite as the growth medium were taken from -80 °C storage and cultured on 1/10 TSA plates for 3 d at 28 °C. A bacterial colony was then scraped from the surface of agar plates into 300 mL half strength TSB. The bacterial isolates were cultured on a rotary shaker and cell growth was assessed in terms of optical density (OD_{660}) using a Thermo Scientific Evolution 60S UV-Visible Spectrophotometer (Thermo Scientific, USA). Bacterial suspensions were concentrated by centrifugation (15 min at 5000 x g) and washed three times within PBS and resuspended in sterile tap water. Finally, the inoculum volume was adjusted to a target concentration of 1×10^8 CFU mL^{-1}.

Six days after emergence, each pea plant was inoculated with 5 mL of antagonistic bacterial suspension delivering 1×10^8 CFU mL^{-1} per plant. After 3 d, pea plants were further inoculated with 5 mL of *A. euteiches* zoospore suspension to deliver 0.5×10^4 zoospore mL^{-1}. The inoculates were applied by injecting the suspensions in the root region using a pipette. The following test controls were: 1) Control A: Pea plants inoculated with 5 mL of antagonistic bacterial suspension at 1×10^8 CFU mL^{-1} per plant only; 2) Control B: Pea plants inoculated with 5 mL of *A. euteiches* zoospore suspension at 0.5×10^4 zoospore mL^{-1} per plant only; 3) Control C: Uninoculated pea plants.

Figure 4.3 Four weeks old pea plant grown in a 500 mL pots containing 500 g of field soil.

4.3.6 Data collection and analysis

Plants were grown for 28 d at which time they were removed from each pot and assessed for the level of disease development. Initially, the roots were washed. The level of disease development was scored and recorded using a 0 to 4 scale (Fig. 4.2) as described in Wakelin et al. (2002): 0 = No symptoms; roots healthy and white; 1 = Initial symptoms of root rot; discoloration, usually a light tan color, in sections of the root system; 2 = Discoloration of most or all the root system, usually still of a tan color. Small watery lesions may be present on the root and around the hypocotyl/epicotyl regions; 3 = Advanced disease symptoms. Dwarfing of the plant and yellowing of the lower leaves. Extensive darkening and discoloration of the root system and extensive lesion formation; 4 = Root entirely rotted / plant dead.

Analysis of variance (ANOVA) (Appendix C) was used to determine the biocontrol activity of the isolates. Moreover, multiple comparisons were made between the controls and each treatment using Statistical Analysis System (SAS version 9.3).

4.4 RESULTS

4.4.1 Cell concentration of candidate bacterial isolates in stock dilution

Cell concentration for the stock culture of the 47 antagonistic bacterial isolates used in growth chamber experiments ranged from a minimum of 1.01 X 10^8 CFU mL^{-1} to a maximum of 1.57 X 10^9 CFU mL^{-1} (Table 4.1). The cell concentration of all isolates was adjusted to a target inoculum concentration of 1 x 10^8 CFU mL^{-1}.

Table 4.1 Cell concentration of the candidate bacterial isolates in stock dilutions.

Isolates	CFU mL^{-1}
K-Hf-L9 (*Pseudomonas fluorescens*)	1.57 X 10^9
K-CB1-1 (*Streptomyces paradoxus*)	1.56 X 10^9
Hf-L2 (*Pseudomonas* spp)	1.50 X 10^9
PCB1-13 (*P. mucidolens*)	1.39 X 10^9
K-Hf-H2 (*Lysobacter capsici*)	1.39 X 10^9
K-CB2-6 (*B. cereus*)	1.32 X 10^9
CB1-11 (*Pseudomonas* spp)	1.28 X 10^9
Ler3-4 (*P. syringae*)	1.26 X 10^9
Hf-L5 (*Pseudomonas* spp)	1.22 X 10^9
K-CB2-4 (*L. antibioticus*)	1.20 X 10^9
Hf-L6 (*Pseudomonas* spp)	1.17 X 10^9
Ler4-2 (*Serratia plymuthica*)	1.15 X 10^9
DR4-4 (*B. thuringiensis*)	1.12 X 10^9
DR1-3 (*Pseudomonas* spp)	1.11 X 10^9
CB3-1 (*P. fluorescens*)	1.10 X 10^9
DR3-1 (*B. thuringiensis*)	1.08 X 10^9
CB1-3 (*L. antibioticus*)	1.08 X 10^9
Ler3-3 (*S. lavendulae*)	1.06 X 10^9
K-Be-H3 (*L. gummosus*)	1.05 X 10^9
PSV1-7 (*Pantoea agglomerans*)	1.04 X 10^9

Continued

Isolates	CFU mL^{-1}
W4-9 (*B. simplex*)	9.85 X 10^8
PCB1-15 (*P.s mucidolens*)	9.84 X 10^8
E3-1 (*Streptomyces* spp.)	9.78 X 10^8
Hf-L4 (*Pseudomonas* spp)	9.23 X 10^8
W2-4 (*B. thuringiensis*)	9.02 X 10^8
(Ler4-1 (*S. plymuthica*)	8.76 X 10^8
Hf-L1 (*Pseudomonas* spp)	7.62 X 10^8
H4-5 (*Pseudomonas* spp)	7.48 X 10^8
MB-H5 (*B. stratosphericus*)	7.38 X 10^8
PK1-11 (*B. atrophaeus*)	7.12 X 10^8
DR1-1 (*Pseudomonas* spp)	6.54 X 10^8
PSV1-9 (*Rhizobium lemnae*)	6.40 X 10^8
H2-7 (*Pseudomonas* spp.)	6.18 X 10^8
PSV1-15 (*Paenibacillus pabuli*)	5.92 X 10^8
NB4-2 (*P. fluorescens*)	5.77 X 10^8
NB2-1 (*Brevibacillus laterosporus*)	5.55 X 10^8
H2-1 (*B. stratosphericus*)	5.44 X 10^8
PCV1-13 (*R. lemnae*)	5.36 X 10^8
DR3-4 (*B. stratosphericus*)	5.28 X 10^8
NB4-3 (*Streptomyces* spp)	4.47 X 10^8
DR1-2 (*S. plymuthica*)	4.12 X 10^8
Ler3-1 (*B. stratosphericus*)	3.99 X 10^8
O1-2 (*Bacillus* spp)	3.78 X 10^8
Ler1-1 (*P. agglomerans*)	3.26 X 10^8
W4-3 (*Paenibacillus polymyxa*)	1.66 X 10^8
H2-5 (*P. polymyxa*)	1.16 X 10^8
Hf-L7 (*Paenibacillus* spp)	1.01 X 10^8

4.4.2 Biocontrol assessment in vermiculite (Trial 1)

Control A (pea plants inoculated with antagonistic bacterial suspension only) and Control C (the negative controls: uninoculated pea plants) showed no disease symptoms and the roots were healthy throughout the experiments. Control B (the positive controls: pea plants inoculated with *A. euteiches* zoospore only) had a root rot score of 2 in each set, indicating significant disease development. Analysis of variance indicated significant differences between the disease score associated with the antagonistic bacterial isolates (Appendix C).

Biocontrol assessment using vermiculite as a growing medium (Trial 1) was conducted in five sets and each set identified a varying number of isolates which significantly ($\alpha = 0.05$) suppressed aphanomyces root rot compared to the control treatments. Relative to the positive control which was inoculated with *A. euteiches* zoospore only, from a total of 47 isolates evaluated for biocontrol activity, with the exception of three isolates namely, K-CB2-6 (*Bacillus cereus*), K-Hf-H2 (*Lysobacter capsici*) and K-CB2-4 (*Lysobacter antibioticus*) that seemed to exacerbate disease, most of the isolates, were identified as having some levels of biocontrol activity. In all five sets, three levels of biocontrol potential were observed at $\alpha = 0.05$ when using vermiculite as a growing medium.

Ten promising antagonistic bacteria were evaluated in each of the first four sets and seven isolates in the fifth set, among these isolates, seven isolates in set 1 (Table 4.2) and 2 (Table 4.3), six isolates in sets 3 (Table 4.4) and 4 (Table 4.5), and three isolates in set 5 (Table 4.6) that had statistically similar biocontrol activity levels in their respective sets. That is, the organisms all reduced the disease level to one or less. In addition, the mean disease level in pea roots involving these isolates was not significantly ($\alpha = 0.05$) different from the control without *A. euteiches* inoculation. Isolates such as DR1-2 (*Serratia plymuthica*) and H4-5 (*Pseudomonas* spp) in set 1;

DR1-3 (*Pseudomonas* spp) and Ler3-1 (*Bacillus stratosphericus*) in set 2; DR4-4 (*B. thuringiensis*) and MB-H5 (*B. stratosphericus*) in set 3; and H2-1 (*B. stratosphericus)*, PCV1-13 (*Rhizobium lemnae*) and H2-5 (*Paenibacillus polymyxa*) in set 4 completely suppressed aphanomyces root rot development in field pea when vermiculite was used as the growing medium.

Biocontrol assessment in vermiculite identified a varying number of isolates in the genera *Pseudomonas* spp., *Streptomyces* spp., *Bacillus* spp. and *Paenibacillus* spp. Others have similarly reported biocontrol properties against several plant pathogens under various conditions for this species (Ganeshan and Manoj, 2005; Srividya et al., 2012; Castillo et al., 2013; Hong et al., 2016).

Based on the result of Trial 1, isolates suppressing aphanomyces root rot to a disease score of at least 1 (i.e., isolates that reduced disease up to initial symptoms of root rot disease) when vermiculite was used as a growing medium were selected for biocontrol assessment in non-sterile soil. The three isolates that seemed to exacerbate disease were also selected for further study in this project. Evaluation of these three isolates may provide additional insight on whether aphanomyces root rot could not only be exacerbated by fungi of the root rot complex discussed in Section 2.5.5 but also by soil bacteria. In the selection process where individual isolates had the same species name according to the 16S rDNA gene sequence and reference strain data obtained from National Center for Biotechnology Information (NCBI) and exhibiting statistically similar biocontrol activity, a single isolate was chosen for further study in this project. As a result, a total of 20 isolates were selected for further study.

Table 4.2 Initial growth chamber screening of bacterial isolates against aphanomyces root rot in field pea grown in vermiculite; set 1.

Antagonistic bacterial isolates	Disease level
Negative control (Control C) †	0.00[d]
DR1-2 (*Serratia plymuthica*)	0.00[d]
H4-5 (*Pseudomonas* spp)	0.00[d]
Hf-L7 (*Paenibacillus* spp)	0.25[cd]
W4-3 (*Paenibacillus polymyxa*)	0.25[cd]
PK1-11 (*Bacillus atrophaeus*)	0.25[cd]
Hf-L2 (*Pseudomonas* spp)	0.50[cbd]
Ler3-4 (*Pseudomonas syringae*)	0.50[cbd]
Ler4-2 (*Serratia plymuthica*)	0.75[cb]
NB4-2 (*Pseudomonas fluorescens*)	1.00[b]
NB2-1 (*Brevibacillus laterosporus*)	1.75[a]
Positive control (Control B)	2.00[a]
LSD = 0.72	

† Negative control: uninoculated pea plants; Positive control: pea plants inoculated with *A. euteiches* zoospore. Plants inoculated with suspensions of each antagonistic bacterial isolates only (Control A) did not manifested visually detectably phytotoxic or phytopathogenic symptoms derived from the antagonistic bacteria. Means across all column followed by same letters are not significantly different at $\alpha = 0.05$ using Fisher Least Significance Difference Test. The data were collected on a 0-4 disease level scale from eight plants in each treatment which were laid out in a complete randomized design in four replicates.

Table 4.3 Initial growth chamber screening of bacterial isolates against aphanomyces root rot in field pea grown in vermiculite; set 2.

Antagonistic bacterial isolates	Disease level
Negative control (Control C) †	0.00[d]
DR1-3 (*Pseudomonas* spp)	0.00[d]
Ler3-1 (*Bacillus stratosphericus*)	0.00[d]
CB3-1 (*Pseudomonas fluorescens*)	0.50[cbd]
Hf-L4 (*Pseudomonas* spp)	0.50[cbd]
(Ler4-1 (*Serratia plymuthica*)	0.50[cbd]
E3-1 (*Streptomyces* spp.)	0.50[cbd]
H2-7 (*Pseudomonas* spp.)	0.50[cd]
PSV1-15 (*Paenibacillus pabuli*)	0.75[cb]
PCB1-13 (*Pseudomonas mucidolens*)	1.00[b]
Positive control (Control B)	2.00[a]
(DR3-1) *Bacillus thuringiensis*	2.00[a]
LSD = 0.60	

† Negative control: uninoculated pea plants; Positive control: pea plants inoculated with *A. euteiches* zoospore. Plants inoculated with suspensions of each antagonistic bacterial isolates only (Control A) did not manifested visually detectably phytotoxic or phytopathogenic symptoms derived from the antagonistic bacteria. Means across all column followed by same letters are not significantly different at $\alpha = 0.05$ using Fisher Least Significance Difference Test. The data were collected on a 0-4 disease level scale from eight plants in each treatment which were laid out in a complete randomized design in four replicates.

Table 4.4 Initial growth chamber screening of bacterial isolates against aphanomyces root rot in field pea grown in vermiculite; set 3.

Antagonistic bacterial isolates	**disease level**
Negative control (Control C) †	0.00[c]
DR4-4 (*Bacillus thuringiensis*)	0.00[c]
MB-H5 (*Bacillus stratosphericus*)	0.00[c]
NB4-3 (*Streptomyces* spp)	0.25[c]
DR1-1 (*Pseudomonas* spp)	0.50[cb]
Hf-L6 (*Pseudomonas* spp)	0.50[cb]
PSV1-7 (*Pantoea agglomerans*)	0.50[cb]
CB1-11 (*Pseudomonas* spp)	1.00[b]
PCB1-15 (*Pseudomonas mucidolens*)	1.00[b]
Positive control (Control B)	2.00[a]
Hf-L1 (*Pseudomonas* spp)	2.00[a]
PSV1-9 (*Rhizobium lemnae*)	2.00[a]
LSD = 0.61	

† Negative control: uninoculated pea plants; Positive control: pea plants inoculated with *A. euteiches* zoospore. Plants inoculated with suspensions of each antagonistic bacterial isolates only (Control A) did not manifested visually detectably phytotoxic or phytopathogenic symptoms derived from the antagonistic bacteria. Means across all column followed by same letters are not significantly different at $\alpha = 0.05$ using Fisher Least Significance Difference Test. The data were collected on a 0-4 disease level scale from eight plants in each treatment which were laid out in a complete randomized design in four replicates.

Table 4.5 Initial growth chamber screening of bacterial isolates against aphanomyces root rot in field pea grown in vermiculite; set 4.

Antagonistic bacterial isolates	Disease score
Negative control (Control C) †	0.00[e]
H2-1 (*Bacillus stratosphericus*)	0.00[e]
PCV1-13 (*Rhizobium lemnae*)	0.00[e]
H2-5 (*Paenibacillus polymyxa*)	0.00[e]
O1-2 (*Bacillus* spp)	0.25[de]
DR3-4 (*Bacillus stratosphericus*)	0.50[dce]
Ler1-1 (*Pantoea agglomerans*)	0.50[dce]
CB1-3 (*Lysobacter antibioticus*)	0.75[dc]
Hf-L5 (*Pseudomonas* spp)	1.00[bc]
W2-4 (*Bacillus thuringiensis*)	1.00[bc]
W4-9 (*Bacillus simplex*)	1.50[ba]
Positive control (Control B)	2.00[a]
LSD = 0.70	

† Negative control: uninoculated pea plants; Positive control: pea plants inoculated with *A. euteiches* zoospore. Plants inoculated with suspensions of each antagonistic bacterial isolates only (Control A) did not manifested visually detectably phytotoxic or phytopathogenic symptoms derived from the antagonistic bacteria. Means across all column followed by same letters are not significantly different at $\alpha = 0.05$ using Fisher Least Significance Difference Test. The data were collected on a 0-4 disease level scale from eight plants in each treatment which were laid out in a complete randomized design in four replicates.

Table 4.6 Initial growth chamber screening of bacterial isolates against aphanomyces root rot in field pea grown in vermiculite; set 5.

Antagonistic bacterial isolates	**Disease score**
Negative control (Control C) †	0.00[c]
K-Be-H3 (*Lysobacter gummosus*)	0.25[cb]
Ler3-3 (*Streptomyces lavendulae*)	0.50[cb]
K-CB1-1 (*S. paradoxus*)	0.75[cb]
K-Hf-L9 (*Pseudomonas fluorescens*)	1.00[b]
Positive control (Control B)	2.00[a]
K-CB2-4 (*Lysobacter antibioticus*)	2.25[a]
K-Hf-H2 (*L. capsici*)	2.50[a]
K-CB2-6 (*Bacillus-cereus*)	2.50[a]
LSD = 0.85	

† Negative control: uninoculated pea plants; Positive control: pea plants inoculated with *A. euteiches* zoospore. Plants inoculated with suspensions of each antagonistic bacterial isolates only (Control A) did not manifested visually detectably phytotoxic or phytopathogenic symptoms derived from the antagonistic bacteria. Means across all column followed by same letters are not significantly different at $\alpha = 0.05$ using Fisher Least Significance Difference Test. The data were collected on a 0-4 disease level scale from eight plants in each treatment which were laid out in a complete randomized design in four replicates.

4.4.3 Biocontrol assessment in soil (Trial 2)

Control C (the negative controls: uninoculated pea plants) and Control B (the positive controls: pea plants inoculated with *A. euteiches* zoospores only) produced mean root rot scores of 0.25 and 3 in each set, respectively. Plants inoculated with suspensions of each antagonistic bacterial isolates only (Control A) did not manifest visually detectable phytotoxic or phytopathogenic symptoms derived from the antagonistic bacteria. Analysis of variance indicated significant differences between the mean disease scores associated with the antagonistic bacteria treatments (Appendix D)

Relative to the positive control, from a total of 20 isolates evaluated for biocontrol activity in the second set of experiments using non-sterile soil (Trial 2), half (50%) of the isolates significantly ($\alpha = 0.05$) suppressed aphanomyces root rot in field pea. Ranking of the isolates differed between the trials conducted in vermiculite (Table 4.2) and soil (Table 4.7). Trial 2 was conducted in two sets and each set identified a varying number of isolates which significantly ($\alpha = 0.05$) suppressed aphanomyces root rot compared to the control treatments (Control B). Although there was variation in biocontrol potential, isolates K-Hf- L9 (*Pseudomonas fluorescens*) and PSV1-7 (*Pantoea agglomerans*) in set 1 (Table 4.7) and isolate K-Hf-H2 (*Lysobacter capsici*) in set 2 (Table 4.8) were identified as having the highest biocontrol effect when used as soil inoculant in Trial 2. Although the level of aphanomyces root rot development was much higher (i.e., ranging from 0.25 to 3.00) compared to Trial 1, all treatments had some level of aphanomyces root rot disease.

Based on the results of biocontrol assessment in non-sterile soil, it is suggested that the top three isolates exhibiting the highest biocontrol effect could be selected for future studies aiming at

identifying the mechanisms by which antagonism is being achieved, identifying the nature (example, volatile or non-volatile) of the active component playing the key role in antagonising *A. euteiches,* and evaluating efficacy under field conditions and approaches of biocontrol agent development.

Table 4.7 Growth chamber biocontrol assessment of bacterial isolates against aphanomyces root rot disease in field pea grown in soil; set 1.

Antagonistic bacterial isolates	**Mean disease level**
Negative control (Control C) †	0.25[f]
K-Hf- L9 (*Pseudomonas fluorescens*)	1.00[c]
PSV1-7 (*Pantoea agglomerans*)	1.00[c]
H2-5 (*Paenibacillus polymyxa*)	1.75[b]
K-CB2-6 (*Bacillus cereus*)	1.75[b]
O1-2 (*B.* sp)	1.75[b]
DR1-2 (*Serratia plymuthica*)	2.00[b]
K-Be- H3 (*Lysobacter gummosus*)	2.00[b]
PCV1-13 (*Rhizobium lemnae*)	2.00[b]
CB3-1 (*P. fluorescens*)	3.00[a]
Ler3-3 (*Streptomyces lavendulae*)	3.00[a]
Positive control (Control B)	3.00[a]
LSD = 0.46	

† Negative control: uninoculated pea plants; Positive control: pea plants inoculated with *A. euteiches* zoospore. Means across all column followed by same letters are not significantly different at $\alpha = 0.05$ using Fisher Least Significance Difference Test. The data were collected on a 0-4 disease level scale from eight plants in each treatment which were laid out in a complete randomized design in four replicates.

Table 4.8 Growth chamber biocontrol assessment of bacterial isolates against aphanomyces root rot disease in field pea grown in soil; set 2.

Species	Mean disease level
Negative-control (Control C) †	0.25[d]
(K-Hf-H2) (*Lysobacter capsici*)	0.50[d]
Hf-L4 (*Pseudomonas sp.*)	1.50[c]
PK1-11 (*Bacillus atrophaeus*)	1.75[c]
NB4-3 (*Streptomyces sp.*)	1.75[c]
K-CB1-1 (*Streptomyces paradoxus*)	1.75[c]
MB-H5 (*Bacillus stratosphericus*)	2.00[bc]
DR4-4 (*Bacillus-thuringiensis*)	2.00[bc]
H4-5 (*Pseudomonas sp.*)	2.50[ba]
K-CB2-4 (*Lysobacter antibioticus*)	3.00[a]
Ler3-4 (*Pseudomonas-syringae*)	3.00[a]
Positive control (Control B)	3.00[a]
LSD = 0.56	

† Negative control: uninoculated pea plants; Positive control: pea plants inoculated with *A. euteiches* zoospore. Plants inoculated with suspensions of each antagonistic bacterial isolates only (Control A) did not manifested visually detectably phytotoxic or phytopathogenic symptoms derived from the antagonistic bacteria. Means across all column followed by same letters are not significantly different at $\alpha = 0.05$ using Fisher Least Significance Difference Test. The data were collected on a 0-4 disease level scale from eight plants in each treatment which were laid out in a complete randomized design in four replicates.

4.4.4 *In vitro* and *in vivo* assay summary for the isolates used in Trial 2

From a total of 184 antagonistic bacterial isolates identified in this project, three isolates K-Hf-H2 (*Lysobacter capsici*), K-Hf-L9 (*Pseudomonas fluorescens*) and PSV1-7 (*Pantoea agglomerans*) were found to possess the highest biocontrol activity when applied as soil inoculants in pot experiments in growth chamber conditions. The mean mycelial growth inhibition zone for isolate K-Hf-H2 and K-Hf-L9 was 5 mm whereas for isolate PSV1-7 was 1 mm. Isolate K-Hf-L9 and PSV1-7 were able to reduce zoospore germination and subsequent growth when tested *in vitro* on PDA plate when applied as an undiluted stock and 100-fold dilution whereas isolate K-Hf-H2 (*Lysobacter capsici*) was able to reduce zoospore germination and subsequent growth only at a stock concentration. Among these three isolates, isolate K-Hf-L9 completely inhibited zoospore germination both at stock concentration and 100-fold dilutions and had consistent suppression potential in Trial 1 and Trial 2 (Table 4.9). The biocontrol effect of this isolate when applied as a soil inoculant in pot experiment in Trial 2 is shown in Figure 4.4.

Generally, the three isolates having the highest biocontrol effects when used as soil inoculants against aphanomyces root rot in field pea in growth chamber conditions can be considered for future studies aiming at evaluating biocontrol efficacy against aphanomyces root rot in field pea and other susceptible crops in field conditions in Saskatchewan.

Table 4.9 *In vitro* and *in vivo* assay summary for the isolates used in trial 2.

Antagonistic bacterial isolates	*In vitro* assay †						*In vivo* assay	
	Mycelia inhibition (mm)	Rank	zoospore inhibition				Trial 1	Trial 2
			Stock suspension	Rank	100-fold dilutions	Rank		
Negative control	none	none	100	1	100	1	0.00^{g}	0.25^{f}
K-Hf-H2 (*Lysobacter capsici*)	5	9	75	12	0	21	2.50^{a}	0.50^{f}
K-Hf-L9 (*Pseudomonas fluorescens*)	5	9	100	1	100	1	1.00^{ed}	1.00^{e}
PSV1-7 (*Pantoea agglomerans*)	1	19	83	7	75	5	0.5^{efg}	1.00^{e}
Hf-L4 (*Pseudomonas* spp)	3	13	75	12	25	17	0.50^{efg}	1.50^{d}
H2-5) *Paenibacillus polymyxa*)	4	12	83	7	58	8	0.00^{g}	1.75^{cd}
K-CB1-1 (*Streptomyces paradoxus*)	7	4	100	1	33	13	0.75^{ef}	1.75^{cd}
K-CB2- 6 (*Bacillus cereus*)	6	6	100	1	100	1	2.50^{a}	1.75^{cd}
NB4-3 (*Streptomyces* spp)	5	9	75	12	58	8	0.25^{fg}	1.75^{cd}
O1-2 (*Bacillus* spp)	3	13	75	12	50	11	0.25^{fg}	1.75^{cd}
PK1-11 (*Bacillus atrophaeus*)	1	19	83	7	17	18	0.25^{fg}	1.75^{cd}
DR1-2 (*Serratia plymuthica*)	6	6	100	1	100	1	0.00^{g}	2.00^{c}
DR4-4 (*Bacillus thuringiensis*)	3	13	75	12	33	13	0.00^{g}	2.00^{c}
K-Be- H3 (*Lysobacter gummosus*)	10	2	100	1	67	6	0.25^{fg}	2.00^{c}
MB-H5 (*Bacillus stratosphericus*)	3	13	75	12	33	13	0.00^{g}	2.00^{c}
PCV1-13 (*Rhizobium lemnae*)	2	18	67	20	33	13	0.00^{g}	2.00^{c}
H4-5 (*Pseudomonas* spp)	3	13	75	12	17	18	0.00^{g}	2.50^{b}
CB3-1 (*Pseudomonas fluorescens*)	8	3	83	7	58	8	0.50^{efg}	3.00^{a}
K-CB2- 4 (*Lysobacter antibioticus*)	12	1	67	20	50	11	2.25^{ba}	3.00^{a}
Ler3-3 (*Streptomyces lavendulae*)	6	6	75	12	17	18	0.50^{efg}	3.00^{a}
Ler3-4 (*Pseudomonas syringae*)	7	4	83	7	67	6	0.50^{efg}	3.00^{a}
Positive control	0	21	0	22	0	21	2.00^{bac}	3.00^{a}

† The *in vitro* assays were *A. euteiches* mycelia and zoospore germination inhibitions. Mycelia inhibition is expressed in mm and each number is the average of six interaction zones that is rounded off to the nearest digit. Zoospore germination inhibition was determined using a 0-3 scale and scores were a total eight observations (i.e., microscopy fields) from two assay plates for each dilution. The *in vivo* assays were the subsequent growth chamber trials in vermiculite (Trial 1) and non-sterile soil (Trial 2), means across all column followed by same letters are not significantly different at $\alpha = 0.05$ using Fisher Least Significance Difference Test. LSD for Trial 1 = 0.68; and Trial 1 = 0.50.

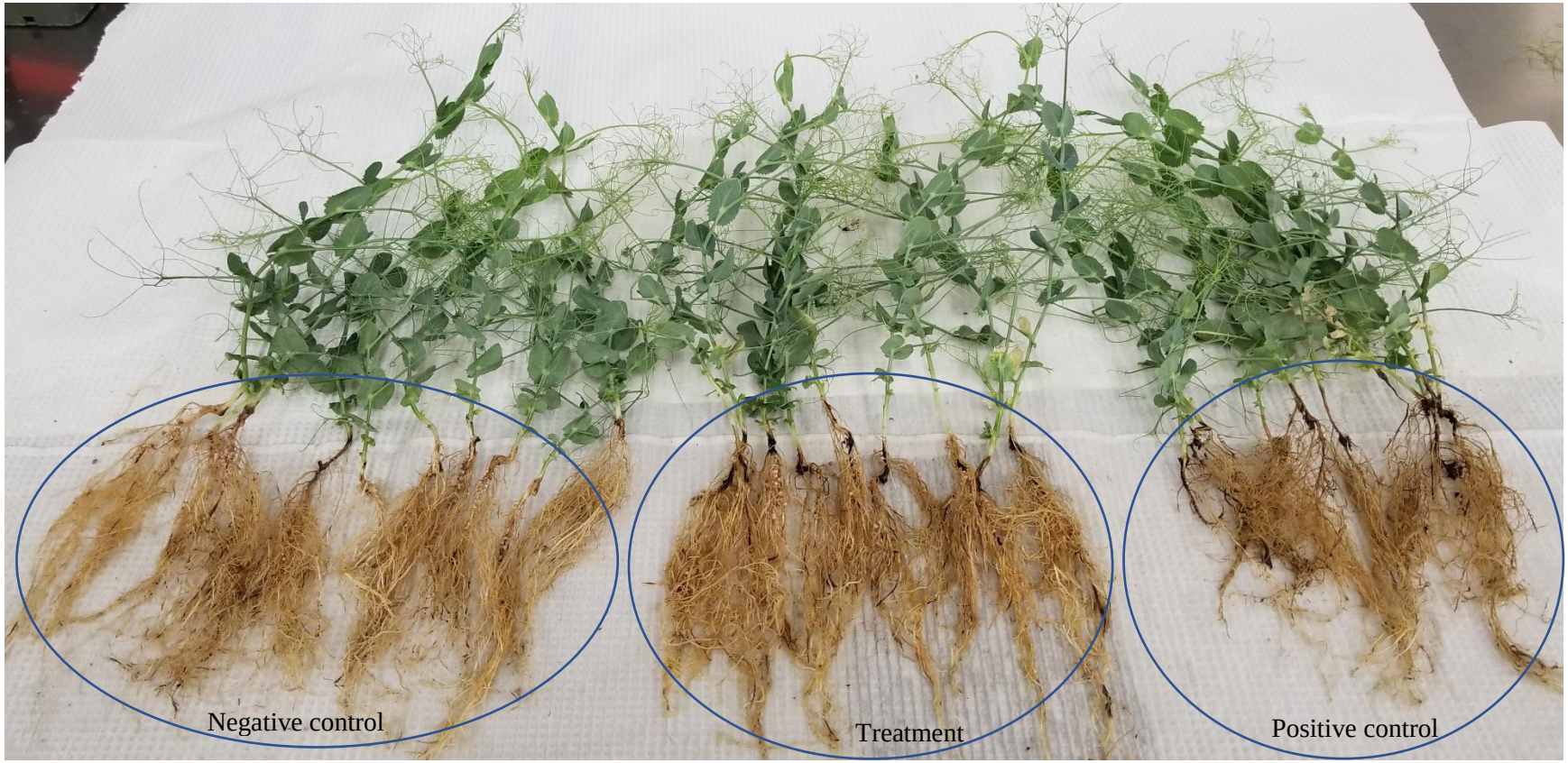

Figure 4.4 Isolate K-Hf-L9 (*Pseudomonas fluorescence*) suppressed aphanomyces rot root when used as soil inoculant in field peas grown in growth chamber conditions. Negative control: roots healthy; mean disease level = 0.25. Treatment: an initial symptom of root rot (light tan colour) Mean disease level = 1. Positive control: Advanced darkening and discolouration; low root mass; mean disease level = 3.

4.4.5 Phylogenetic analysis of isolates used for biocontrol assessment

Antagonistic bacterial isolates identified as belonging to the same species were found to be close phylogenetic neighbors of each other (Fig. 4.5). The circular phylogenetic tree was constructed based on the 16S rDNA gene sequences using the maximum likelihood method in MEGA software package version 7 (Tamura et al., 2011). The tree shows the relationships among the different antagonistic bacterial isolates, and the biocontrol activity played by these isolates when used as bioinoculants against aphanomyces root rot in field peas grown in growth chamber conditions. The specific biocontrol is indicated by different colors. Species displayed in maroon color were isolates that suppressed aphanomyces root rot in field pea grown in both vermiculite and soil whereas species displayed in navy and lime colors were isolates that only suppressed aphanomyces root rot in field peas grown in vermiculite and soil, respectively.

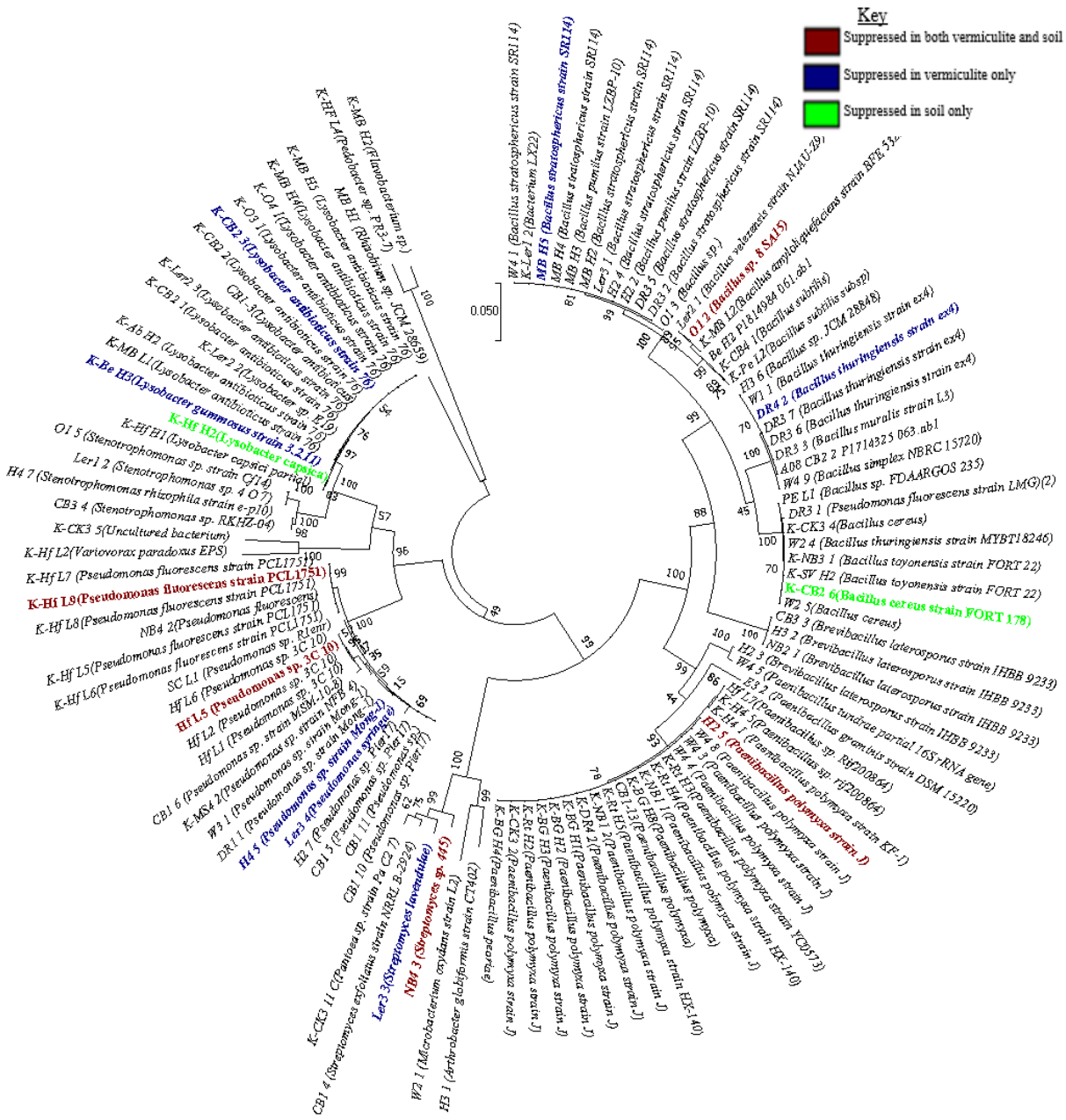

Figure 4.5 Circular Phylogenetic Tree based on 16S rDNA gene sequences showing the relationships among the different antagonistic bacteria that inhibited *Aphanomyces euteiches* growth under laboratory and growth chamber conditions. Trees were constructed by the maximum likelihood method using MEGA version 7 (Tamura et al., 2011). Species names are displayed horizontally in clockwise direction starting at an angle 0. Center hole (20%), estimated substitution expected number of changes per site.

4.5 DISCUSSION

Soilborne diseases such as aphanomyces root rot, for which management is a challenge because of insufficient or ineffective control options, continue to impose significant crop losses both in productivity and quality throughout the world (Spies, 2008). Disease management strategies that utilize antagonistic bacteria are one of the alternatives to manage aphanomyces root rot in field pea. Several antagonistic bacterial species of *Pseudomonas*, *Streptomyces* and *Bacillus* have been successfully utilized as biocontrol agents of various soilborne pathogens (Salaheddin et al., 2010; Srividya et al., 2012; Castillo et al., 2013; Khabbaz et al., 2013; Hong et al., 2016). The majority of these antagonistic bacteria are common soil inhabitants which may already be playing a role in biocontrol activities towards plant pathogens (Santoyo et al., 2012; Khabbaz et al., 2013).

In this study, bacterial isolates antagonistic to *A. euteiches* mycelia and zoospore growth stages were isolated and identified using *in vitro* assays from soil samples collected from commercial field pea fields across Saskatchewan (i.e., *in vitro* assays, Chapter 3). In the subsequent growth chamber trials, laboratory produced *A. euteiches* zoospores were used as the source of pathogen inoculum. This approach was intended to provide reproducible levels of aphanomyces root rot pressure, instead of using plant growth media that is naturally infested with pathogen oospores. Biocontrol efficacy was measured based on visual assessment of aphanomyces root rot development; thus, variability that may exist between different ratings can be considered as a limitation of visual disease assessment as it was similarly stated by Mutka and Bart (2015).

4.5.1 Biocontrol assessment in vermiculite (Trial 1)

The initial growth chamber-based *in vivo* biocontrol assessment that used vermiculite as the growth medium, was designed to screen isolates with potential to control or reduce aphanomyces

root rot in field pea. Because aphanomyces root rot development is often associated with high moisture content, it was important to find potential biocontrol isolates with tolerance to high moisture level that grow and proliferate in conditions conducive to the pathogen. Thus, the water holding capacity of each pot containing vermiculite was maintained at 80%.

Vermiculite is a soil-less medium that enables water retention and rapid separation of roots with minimal damage and discoloration to the root system. The other advantage of using vermiculite was that there was no introduction of other pathogens which may confound the efforts to determine aphanomyces root rot score in each treatment. Therefore, it was possible to measure the biocontrol efficacy of the antagonistic bacterial isolates alone against aphanomyces root rot pressure. This was further substantiated by the negative control (Control C) treatment in this study which did not show any root rot symptoms in all rounds of the biocontrol assessment in vermiculite. These negative control treatments were not inoculated with any *A. euteiches* zoospore or antagonistic bacteria.

The *Lysobacter* strains identified in this project showed a very strong *in vitro* mycelia growth inhibition; however, there was inconsistent and for some isolates poor or no-biocontrol activity was observed when evaluated *in vivo* using vermiculite as a growing media. A similar result was obtained by Gómez et al. (2015) in a study conducted to understand the diversity and activity of *Lysobacter* spp. According to Gómez et al. (2015) such an inconsistency could be related to competitiveness and colonization potential. As noted earlier in this study, considering the value of colonization for biocontrol activity, the inconsistency in biocontrol potential by the *Lysobacter* species may be due to the lack of competitiveness in the rhizosphere of the field pea root leading to poor or no-colonization. Usually chemotaxis and active motility towards nutrients released by a host plant as root exudates represent the first steps in rhizosphere colonization (De Weert and

Bloemberg, 2006). Although various factors determine motility of microorganisms, the presence or absence of locomotory structures like flagella plays a pivotal role and makes the movement of the microorganisms active or passive. Gómez et al. (2015) indicated that 18 *Lysobacter* strains which were identified to be *L. antibioticus, L. gummosus*, *L. capsici* and *L. enzymogens* did not possess flagella and the absence of flagella were considered to limit competitiveness in the rhizosphere. In the present study, *A. euteiches* zoospores have flagella and vermiculite is less compact compared to natural soil which both may give a competitive advantage to the pathogen as compared to *Lysobacter*.

Based on the result of the current study, a total of 20 antagonistic bacterial isolates were selected for further growth chamber-based biocontrol assessment in non-sterile agricultural soil. The 20 selected isolates constitutes five isolates from *Bacillus* spp. and *Pseudomonas* spp. three isolates from *Streptomyces* spp. and *Lysobacter* spp., one isolate from *Pantoea* spp., *Paenibacillus* spp., *Serratia* spp., and *Rhizobium* spp. Except for the three isolates, two from *Lysobacter* spp. and one from *Bacillus* spp. which appeared to exacerbate disease as described before, the rest of the antagonistic bacterial isolates were selected based on biocontrol potential towards aphanomyces root rot in field pea grown in vermiculite. This selection, with higher number of isolates from *Bacillus*, *Pseudomonas* and *Streptomyces*, was consistent with reports by Khabbaz et al. (2013) and Hong et al. (2016) which stated that several antagonistic bacterial species of *Bacillus*, *Pseudomonas* and *Streptomyces* have been successfully utilized as biocontrol agents of plant diseases.

Compared to *in vitro* assays based on microbial culture media, growth chamber trials were a more rigorous test of the biocontrol agent assessment, and some bacteria that showed potential in the biocontrol of *A. euteiches* mycelia and zoospore *in vitro*, failed to inhibit disease development

in vivo. This could be because of interactions with factors such as organic matter, pH, nutrient and moisture level of the growing media, as stated by Law et al. (2017). Moreover, some isolates identified to be the same species exhibited different biocontrol efficacy, suggesting that strain specificity was an influencing factor. Similarly, Thomas and Upreti (2014) observed inconsistent biocontrol activity among three isolates of *Bacillus pumilus*.

In this study, the mechanism of biocontrol activity exhibited by the candidate antagonistic bacterial isolates was not investigated. However, several mechanisms of antagonism such as competition, antibiotics, production of siderophores and hydrogen cyanide, parasitism and induction of systemic resistance have been proposed for other biocontrol agents of various soilborne diseases (Pereg and McMillan, 2015). The biocontrol activity of the antagonistic bacterial isolates may not be restricted to only one mechanism; thus, an efficient biocontrol agent may utilize combinations of multiple mechanisms resulting into a pooled synergistic effect and inhibit plant pathogens (Anith et al., 2003). Root colonization by the introduced biocontrol agent may also play an important role in biological control efficacy (Hass and Defago, 2005).

4.5.2 Biocontrol assessment in soil (Trial 2)

A second growth chamber-based biocontrol assessment in non-sterile agricultural soil was conducted in a manner similar to the biocontrol assessment conducted using vermiculite. Candidate biocontrol agents were selected based on antagonistic potential *in vitro* assays and aphanomyces root rot disease suppression potential in initial growth chamber trials (Sections 3.4.3; 3.4.4 and 4.4.2).

The presence of root rot symptoms in all the treatments and the higher level of aphanomyces root rot development in the positive control (Control B) treatments compared to the biocontrol

assessment in vermiculite could be attributed to the presence of a root rot disease causing pathogen including *Aphanomyces* in the soil used to grow the field peas. The presence of root rot symptom similar to aphanomyces root rot may have been a confounding factor. Pea root rot symptoms caused by fungi of the root rot complex are usually quite similar in field conditions, although disease symptom development depends on environmental factors such as relative levels of inoculum and the levels and types of genetic resistance in field pea, *A. euteiches* often causes disease in a complex involving other pathogens (Hughes and Grau, 2013).

The isolates varied in the level of biocontrol achieved when evaluated in either vermiculite and agricultural soil, presumably due to differences in chemical and physical properties of the growing media as well as differences in the absence and presence of other microorganisms. Future studies could repeat these experiments to confirm the nature of the observed variability and validate these preliminary results.

Although there was variation in biocontrol potential among isolates, from the total of 20 antagonistic bacterial isolates evaluated in two rounds, isolates such as K-Hf- L9 (*P. fluorescens*), PSV1-7 (*Pantoea agglomerans*) and K-Hf-H2 (*L. capsici*) were identified as having the highest biocontrol effect when used as soil inoculant. Different strains of the same bacterial species have been studied as biocontrol agents of several plant diseases (Jayaraj et al., 2007; Park et al., 2008 and Rezzonico et al., 2009).

Isolate K-Hf-L9 is one of the three best candidate biocontrol agents that supressed aphanomyces root rot both *in vitro* and *in vivo* assays. This isolate was obtained from field soil collected at Holdfast, from a low spot of the field. The cropping history of the field was pea in 2013, flax in 2014, canola in 2015 and lentil in 2016. This suggests that the isolate may be compatible with these crops and may also suppress aphanomyces root rot in lentil.

Pseudomonas fluorescens are rod-shaped non-pathogenic saprophytic bacteria that colonize various environments including soil, water and plant surfaces (Ganeshan and Manoj, 2005). *Pseudomonas fluorescens* produces fluorescein which is a soluble greenish fluorescent pigment (Silva et al., 2006). Except for a few strains of *P. fluorescens* which use NO_3 as an electron acceptor in place of O_2, they are obligate aerobes (Kumar et al., 2016). Several strains of *P. fluorescens* are known to improve plant growth and suppress different diseases (Ganeshan et al., 2005). For example, Jayaraj et al. (2007) identified a *P. fluorescens* strain from the rhizosphere of tomato which was highly antagonistic to *Pythium aphanidermatum* both *in vitro* and *in vivo*. *Pythium aphanidermatum is* a causative agent for damping-off disease in tomato. Choi et al. (2006) indicated that strains of *P. fluorescens* pc78 and mc75 inhibited plant pathogenic fungi *in vitro* and the same strains suppressed rice sheath blight when evaluated *in vivo*.

Isolate PSV1-7 (*Pantoea agglomerans*) was acquired from existing bacteria library (courtesy Dr. J. Germida, University of Saskatchewan) and was previously isolated from interior region of pea roots. The isolate supressed aphanomyces root rot both *in vitro* and *in vivo* assays. *Pantoea agglomerans* is a Gram-negative bacterium that belongs to the family of *Enterobacteriaceae* (Rezzonico et al., 2009). Strains of *P. agglomerans* have been studied as biological control agents against various plant diseases including fungal and bacterial diseases. For instance, a strain of *P. agglomerans* was used as a biological control agent against post-harvest fungal diseases (Bonaterra et al., 2005) and the bacterial disease of barley, basal kernel blight (Braun-Kiewnick et al., 2000). Additionally, strains of *P. agglomerans*, such as E325, P10c and C9-1 have been commercialized for use against *Erwinia amylovora*, the causative agent for fire blight on pear (Pusey, 2002; Johnson et al., 2004; Stockwell et al., 2010). Rezzonico et al. (2009) reported that several strains of *P.* agglomerans are sold as commercial biological control agents against the fire blight pathogen.

For example, *P. agglomerans* is commercially available for use in USA and Canada (Bloomtime Biological™, BlightBan C9-1™) and New Zealand (BlossomBless™) (Braun-Kiewnick et al., 2012). Although some strains of *P. agglomerans* were reported to produce antibiotics and other bioactive molecules (Pusey et al., 2008), the primary mode of action is reportedly due to competitive exclusion which involves the occupation of sites otherwise colonized by the pathogen (Braun-Kiewnick et al., 2012). Therefore, the *in vitro* and *in vivo* results observed in the current study coupled with the presence of various prior reports on the potential use of *P. agglomerans* as biological control agents of various plant disease. This supports the potential promise that the isolate will be effective under field conditions and may also suppress aphanomyces root rot in other susceptible crops.

K-Hf-H2 (*Lysobacter capsici*) is another isolate identified as having biocontrol activity against aphanomyces root rot in field pea when applied as a soil inoculant. This isolate was obtained from field soil collected at Holdfast, but in a high spot location. The cropping history of the field was pea in 2013, flax in 2014, canola in 2015 and lentil in 2016. During initial biocontrol assessment in vermiculite this isolate not only failed to exhibit biocontrol potential but also appeared to exacerbate disease in the field peas. This could be attributed to the growing medium (i.e., vermiculite) which might have altered the production of inhibitory metabolites by the isolate K-HF-H2 and/or the isolate might need to interact with other microbes to maintain antagonistic potential against *A. euteiches*.

Lysobacter are well-known to produce various extracellular bioactive molecules which include enzymes and antimicrobial compounds with activity against bacteria, fungi, oomycetes, and nematodes (Gómez et al., 2015). Enzymes such as chitinases (Zhang et al., 2001), glucanases (Palumbo et al., 2005), elastases, endonucleases, endoamylases, esterases, keratinases and

phosphatases (Reichenbach, 2006), lipases (Ko et al., 2009) and proteases (Vasilyeva et al., 2014) are reported to be produced by *Lysobacter*. Bioactive compounds such as dihydromaltophilin, lactivicin, lysobactin, maltophilin, phenazine, tripopeptin and xanthobaccin are described as antimicrobial compounds produced by *Lysobacter* (Xie et al., 2012).

Based on the 16S rDNA sequencing, the *Lysobacter* strains identified from the soil samples assessed in this project belonged to *L. antibioticus, L. gummosus* and *L. capsici.* All strains showed a very strong *in vitro* biocontrol activity against *A. euteiches* mycelia growth. When these *Lysobacter* strains were introduced into vermiculite and soil in growth chamber conditions inconsistent aphanomyces root rot suppression potential was observed. Subsequent growth chamber-based biocontrol assessment revealed that K-Hf-H2 was able to significantly suppress aphanomyces root rot when used as soil inoculant. The lack of *in vivo* biocontrol activity for the rest of the isolates is may be attributed to poor colonization of the field pea rhizosphere by the introduced *Lysobacter* strains. This finding was consistent with a research conducted by Gómez et al. (2015). Briefly, Gómez et al. (2015) also observed inconsistent *in vitro* and *in vivo* biocontrol activity when 18 *Lysobacter* strains which were identified to be *L. antibioticus, L. gummosus, L. capsici and L. enzymogens* evaluated against pathogens such as *R. solani*, *Pythium ultimum*, *Aspergillus niger*, *Fusarium oxysporum* and *Xanthomonas campestris*, and consequently speculated that such inconsistency could be due to the lack of competitiveness in the rhizosphere or requirement of interaction with certain groups of microbes.

Several other reports indicated that *Lysobacter* species were able to effectively suppress plant pathogens. Yuen et al. (2001) indicated that *L. enzymogenes* inhibited *Uromyces appendiculatus* and Islam et al. (2005) reported that *Lysobacter sp*. strain SB-K88 inhibited *Aphanomyces cochlioides* in sugar beet and spinach. Moreover, a report by Lee et al. (2014) indicated that *L.*

capsici YS1215 was able to limit root-knot disease caused by *Meloidogyne incognita*. Park et al. (2008) identified a strain of *L. capsici* (*L. capsici* sp. nov.) which inhibited mycelia growth of *Pythium ultimum, Colletotrichum gloeosporioides, Fusarium oxysporum, Botrytis cinerea, Rhizoctonia solani* and *Botryosphaeria dothidea*.

Overall, the results of this study indicated that *Lysobacter* species showed a high variability in biocontrol activity against *A. euteiches* mycelia, zoospore and *in vivo* assessments. Moreover, some strains of *Lysobacter* species failed to exhibit biocontrol activity when assessed *in vivo* using sterile vermiculite; however, these strains exhibited biocontrol activity when used in soil. Based on the current result, the lack of biocontrol efficacy by some isolate in vermiculite could be due to the physicochemical properties of the growing medium and/or the isolates may require interactions with specific groups of microorganisms to exhibit biocontrol activity as speculated by Gómez et al. (2015) and suppress aphanomyces root rot in field pea.

4.6 CONCLUSIONS

The present study is one of the few studies conducted to develop biological control agents of aphanomyces root rot in pea and although attempts have been made to use strains of *Pseudomonas fluorescens* (Xu et al., 1986), this work is most likely the first to describe the use of *Pantoea agglomerans* and *Lysobacter capsici* as biological control agents of aphanomyces root rot in pea. Following application, biocontrol agents are exposed to various biotic factors which include competition and predation, and abiotic factors such as changes in temperature, osmolarity, pH, availability of nutrients and water. Thus, these factors determine the success of biocontrol agents and often tend to contribute to variability in biocontrol potential (Cañamás et al., 2009).

One of the major limitations to utilizing microorganisms such as K-Hf-L9, PSV1-7 or K-Hf-H2 as biocontrol agents would be the ability of the isolates to control disease under a range of soil and environmental conditions. As a result, to fully understand the range of the isolates ecological niche, further studies aimed at exploring the ecology of these three isolates need to be undertaken. Nevertheless, reports in the literature indicate that for some strains of *Pseudomonas fluorescens* (Lejbølle, 2004) the most critical factors affecting their growth in soil were dry conditions and hyperosmolarity whereas pH and temperature were reported to be the most critical factors affecting the survival of *Pantoea agglomerans* (Cañamás et al., 2009) and *Lysobacter capsici* (Segarra et al., 2015), respectively.

In conclusion, isolates K-Hf-L9, PSV1-7 and K-Hf-H2 described in this thesis have a clear potential for the biocontrol of aphanomyces root rot in field pea in growth chamber conditions. To exploit the potential of these biocontrol candidates, future studies such as evaluation of the candidate biocontrol agents in different soil types, and efficiency testing in combination with

fungicides can be considered. Different application methods such as soil inoculation, seed coat application can be further explored. Soil inoculation can be either by mixing of the biological control agent with soil or applying it on the seedrow (Vasudevan et al., 2002). Seed coat application, which involves dipping seeds in inoculant culture or mixing the seeds with the biological control agent using wetting agents (Yang et al., 2008) should be assessed. Suitable application methods are anticipated to assist the success of biological control agents in suppressing plant pathogens.

In addition to the antagonistic bacterial isolates identified in this study, future studies can focus on other plant-growth promoting rhizobacteria for biocontrol activity against aphanomyces root rot in field pea. For example, several rhizobial strains such as *R. leguminosarum* bv. trifolii, *R. leguminosarum* bv. viciae, *R. meliloti* and *R. trifolii* have been reported to secrete antibiotics and cell-wall degrading enzymes that can inhibit phytopathogens (Gopalakrishnan et al., 2015). Moreover, rhizobial strains also exhibit other biocontrol mechanisms and limit the growth of pathogens. Such mechanisms include competition for available nutrients like iron by producing high affinity siderophores (Arora et al., 2001). Such consideration may enable the identification of biocontrol agents with dual benefits.

5. SYNTHESIS AND CONCLUSION

The overall objective of this study was to evaluate the potential for biological control of aphanomyces root rot in field pea in Saskatchewan conditions. Development of an efficient *in vitro* screening method that delivers reproducible and consistent results in a relatively shorter period was an essential first step to isolate and identify potential bacteria antagonistic to *A. euteiches*.

In vitro crowded plate assays, dual plate assays and zoospore germination inhibition assays were undertaken as preliminary screening criterion for antagonism, isolation and identification of bacterial isolates with biocontrol attributes against *A. euteiches*. The zone of inhibition and percent of zoospore germination inhibition were considered as a measure of antagonistic potential of the isolates. In this study, since it was anticipated that a lot of time and energy could be saved in the course of developing efficient biocontrol agents, an intermediate *in vivo* based screening approach was used. The *in vivo* assays were conducted on the basis of interaction of the factors involved in disease causation such as host, pathogen and environment which are components of the disease triangle (Francl, 2001); thus, the *in vivo* assay more closely resembled interactions expected under field conditions. A screening method that considers host plant, pathogen and environment is anticipated to produce a more representative picture than the *in vitro* assays based on microbial culture media. As a result, two types of growth chamber-based biocontrol assessments that utilized vermiculite and soil as the field peas growing media were conducted.

In vitro screening identified a total of 184 antagonistic bacterial isolates exhibiting biocontrol against *A. euteiches*, of which 22 were from a previously existing bacteria culture collection and the rest were isolated from soil samples collected from pea fields in Saskatchewan. The mean mycelial growth inhibition zone ranged from 1 mm to 12 mm. The maximum inhibition zone was

recorded for isolate K-CB2-4 (*Lysobacter antibioticus*). The genus *Lysobacter* constitutes the non-spore forming Gram-negative bacteria which has antagonistic effects against phytopathogens, and bacterial strains of this genus are considered as potential candidate for biological control of crop disease (Hayward et al., 2010; Qian et al., 2012). For example, *L. antibioticus* HS124 produce 4-hydroxyphenylacetic acid and several lytic enzymes which have biocontrol activity against Phytophthora blight (Ko et al., 2009). Chowdhury et al. (2012) also indicated that myxin produced by *L. antibioticus* blocks DNA synthesis of pathogens. Myxin is a phenazine di-N-oxide that displays potent antibiotic activity against a variety of organisms under aerobic conditions and it was first isolated from *Sorangium* sp. by Peterson et al. (1966). Strain of *Lysobacter* species (*L. antibioticus* 13-1) was reported to be a potential biocontrol agent for rice bacterial blight and has been shown to reduce disease incidence up to 78 percent (Ji et al., 2008; Chowdhury et al., 2012).

The mean zoospore germination inhibition ranged from 0 to 100 % when bacterial cultures were applied as either undiluted stock culture or when applied as 100-fold. DR1-2 (*Serratia plymuthica*), DR3-4 (*Bacillus stratosphericus*), K-CB2-6 (*Bacillus cereus*) Ler1-1 (*Pantoea agglomerans*), and K-Hf-L9 (*Pseudomonas fluorescens*) were among the isolates that exhibited complete inhibition of zoospore germination when applied as both a stock and 100-fold dilutions. Several strains of *Bacillus, Pseudomonas, Pantoea* and *Serratia* have been used as biocontrol agents of various plant diseases under different conditions (Heungens et al., 2001; Ganeshan et al., 2005; Rezzonico et al., 2009; Wang et al., 2003). For example, Wakelin et al. (2002) identified spore forming *Bacillus* strains that suppressed *A. euteiches* both *in vitro* and *in vivo* conditions.

Based on the 16S rDNA sequencing, the antagonistic bacterial isolates identified in this project were placed into 18 different genera. *In vitro* identification of isolates inhibitory to *A. euteiches* growth stages is an indication that the pathogen lifecycle can be interrupted. Variations

of inhibition potential and diverse identity among isolates suggests that the mechanisms by which biocontrol was achieved such as the production and secretion of inhibitory compounds and/or the mode of action exerted by the inhibitory metabolites likely varies between isolates.

In this study, the mechanism of biocontrol activity exhibited by the antagonistic bacterial isolates was not investigated. However, several mechanisms of antagonism such as competition for nutrients and space, production of antibiotics, toxins, siderophores, hydrogen cyanide or host cell wall degrading enzymes (chitinases, proteases, lipases) as well as parasitism and induction of systemic resistance have been proposed for other biocontrol agents of various soilborne plant diseases (Krechel et al., 2002; Pereg and McMillan, 2015). Moreover, biocontrol activity may not be restricted to only one mechanism; thus, biocontrol agents may utilize combinations of multiple mechanisms resulting into a pooled synergistic effect and inhibit plant pathogens (Anith et al., 2003). In the process of controlling soilborne plant root diseases, the role of root colonization potential by the introduced biocontrol organism can not be overlooked (Hass and Defago, 2005). Based on the anti-pathogen interactions observed *in vitro* assays in this project, it is speculated that production of inhibitory metabolites which may include antibiotics, toxins, hydrogen cyanide and hydrolytic enzymes might have been involved in the direct inhibition of pathogen growth through competitive antagonism.

In vivo biocontrol assessment of aphanomyces root rot in field pea grown in vermiculite evaluated 47 antagonistic bacteria which were most effective and consistently inhibited of *A. euteiches* mycelia and zoospore growth stages in the *in vitro* assays. Most of the isolates evaluated exhibited biocontrol activity in a set of growth chamber trials; however, isolates that failed to exhibit biocontrol activity and that appeared to exacerbate disease were also observed. This could be attributed to the growing medium (i.e., vermiculite) which might have altered the production of

inhibitory metabolite and/or the isolate might need to interact with other microbes to maintain antagonistic potential against *A. euteiches*.

In vivo growth chamber-based biocontrol assessments of aphanomyces root rot in field pea grown in non-sterile soil evaluated 20 antagonistic bacterial isolates which suppressed aphanomyces root rot disease. The three isolates which appeared to exacerbate disease when evaluated in vermiculite were also included. Compared to the biocontrol assessment in vermiculite the level of aphanomyces root rot development was much higher when non-sterile field soil was used as a field pea growing medium and all treatments had some level of aphanomyces root rot. This could be attributed to the presence of a root rot causing pathogen including *Aphanomyces* in the soil. Isolate K-Hf-H2 (*Lysobacter capsici*) that appeared to exacerbate disease when evaluated in vermiculite was able to significantly suppress aphanomyces root rot when used as soil inoculant in growth chamber conditions suggesting that the isolate most likely need to interact with other microbes to maintain antagonistic potential against *A. euteiches*.

Compared to *in vitro* assays based on microbial culture media, growth chamber trials were a more rigorous test of the biocontrol agent assessment and to some extent the preliminary screenings for biocontrol activity predicted the potential for biological control of aphanomyces root rot *in vivo*. In this study, the biocontrol assessment in vermiculite was an essential step in the screening process. Using vermiculite as the growth medium enabled high-water retention and rapid separation of roots with minimal damage and discoloration to the root system. Moreover, although three isolates out of 47 seemed to exacerbate disease, the two candidate biocontrol isolates (K-Hf-L9 and PSV1-7) which were identified as having the highest biocontrol efficacy in soil were also identified in this screening step. Overall three isolates K-Hf-L9 (*P. fluorescens*), PSV1-7 (*P. agglomerans*) and K-Hf-H2 (*L. capsici*) were identified as having the highest biocontrol potential

against aphanomyces root rot in field pea when applied as cell suspension at the point of plant emergence. Bacterial strains of the same species have been studied as biocontrol agents of plant diseases (Islam et al., 2005; Jayaraj et al., 2007; Rezzonico et al., 2009).

The present study has offered evidence that aphanomyces root rot can be controlled using biocontrol agents. Therefore, further studies aimed at assessing the efficacy and other manipulative studies that would maximize biocontrol potential and effective utilization of the candidate biocontrol agents such as K-Hf-L9 (*P. fluorescens*), PSV1-7 (*P. agglomerans*) and K-Hf-H2 (*L. capsici*) need to be conducted under field conditions in Saskatchewan. However, several studies indicated the existence of inconsistent biocontrol efficacy (i.e., being less effective or completely ineffective) when biocontrol products are introduced under commercial field conditions even though very good efficacy was observed in controlled conditions (Bardin et al., 2015). Factors such as climatic variations (i.e., temperature, humidity and radiation) encountered in field conditions, a lack of ecological competence (i.e., survival and colonization ability) of the biocontrol agent, intrinsic traits of the antagonistic microbe (i.e., variable production of required metabolites or enzymes) and/or an unstable quality of the formulated product usually account for biocontrol efficacy variability (Ruocco et al., 2011; Bardin et al., 2015).

.

REFERENCES

Adriano, D.C., N.S. Bolan, and C.D. Barton. 2005. Root exudates and microorganisms. Elsevier 421-428.

Agriculture Agri-Food Canada. 2017. Crop profile for field pea in Canada. http://publications.gc.ca/collections/collection_2017/aac-aafc/A118-10-3-2015-eng.pdf

Agriculture and Agri-Food Canada. 2005. Crop profile for field pea in Canada. http://publications.gc.ca/collections/collection_2009/agr/A118-10-3-2005E.pdf

Altermatt, F., E.A. Fronhofer, A. Garnier, A. Giometto, F. Hammes, J. Klecka, D. Legrand, E. Maechler, T.M., Massie, F. Pennekamp, and M. Plebani. 2015. Big answers from small worlds: a user's guide for protist microcosms as a model system in ecology and evolution. Methods in Ecol. and Evol. 6(2): 218-231.

Anith, K.N., N.V. Radhakrishnan, and T.P. Manomohandas. 2003. Screening of antagonistic bacteria for biological control of nursery wilt of black pepper (Piper nigrum). Microbiol. Res. 158(2): 91.

Arora N.K., S.C. Kang, and D.K. Maheshwari. 2001. Isolation of siderophore producing strains of *Rhizobium meliloti* and their biocontrol potential against *Macrophomina phaseolina* that causes charcoal rot of groundnut. Curr. Sci. 81: 673–677.

Baan, C.D., M.C.J. Grevers, and J.J. Schoenau. 2009. Effects of a single cycle of tillage on long-term no-till prairie soils. Can. J. Agric. Sci. 89(4): 521-530.

Bais, HP, S.W. Park, T.L. Weir, R.M. Callaway, J.M. Vivanco. 2004. How plants communicate using the underground information superhighway. Trends Plant Sci. 9: 26-32.

Banerjee, G., S. Gorthi, and P. ChattoPadhyay .2018. Beneficial effects of bio-controlling agent *Bacillus cereus* IB311 on the agricultural crop production and its biomass optimization through response surface methodology. An. Acad. Bras. Cienc. 90(2): 2149-2159.

Banniza, S., V. Bhadauria, C.O. Peluola, C. Armstrong-Cho, and R.A.A. Morrall. 2013. First report of Aphanomyces euteiches in Saskatchewan. Can. Plant Dis. Surv, 93: 163-164.

Bardin, M., S. Ajouz, M. Comby, M. Lopez-Ferber, B. Graillot, M. Siegwart, and P.C. Nicot. 2015. Is the efficacy of biological control against plant diseases likely to be more durable than that of chemical pesticides? Front. Plant Sci. 6: 566.

Bienkowski, D. 2012. Biological control of *Rhizoctonia* diseases of potato. Ph.D. diss., Lincoln University.

Bonaterra, A., J. Camps, and E. Montesinos. 2005. Osmotically induced trehalose and glycine betaine accumulation improves tolerance to desiccation, survival and efficacy of the postharvest biocontrol agent *Pantoea agglomerans* EPS125. FEMS Microbiol. Lett. 250, 1-8.

Bouhot, D. 1979. Soil sickness: study of celery root necrosis. B-experimental results. French. Pepinieristes Horticulteurs Maraichers.

Bowers, J.H., and J.L. Parke. 1993. Epidemiology of *Pythium* damping-off and aphanomyces root rot of peas after seed treatment with bacterial agents for biological control. *Phytopathology*, 83(12): 1466-1473.

Braun-Kiewnick, A., A. Lehmann, F. Rezzonico, C. Wend, T.H. Smits, and B. Duffy. 2012. Development of species, strain and antibiotic biosynthesis specific quantitative PCR assays for *Pantoea agglomerans* as tools for biocontrol monitoring. J. Microbiol. Methods 90(3): 315-320.

Canadian Grain Commission. 2017. Quality of western Canadian peas production. https://www.grainscanada.gc.ca/peas-pois/harvest-recolte/2017/pdf/hqp17-qrp17-en.pdf

Cañamás, T.P., I. Viñas, M. Abadias, J. Usall, R. Torres, and N. Teixidó. 2009. Acid tolerance response induced in the biocontrol agent Pantoea agglomerans CPA-2 and effect on its survival ability in acidic environments. Microbiol. Res. 164(4): 438-450.

Canola council of Canada. 2017. https://www.canolacouncil.org/canola-encyclopedia/field-characteristics/effects-of-soil-characteristics/

Castillo, H.F., C.F. Reyes, G.G. Morales, R.R. Herrera, and C Aguilar. 2013. Biological control of root pathogens by plant-growth promoting *Bacillus* spp. In weed and pest control-conventional and new challenges. InTech. 1-26.

Cavaglieri, L., J. Orlando, and M. Etcheverry. 2009. Rhizosphere microbial community structure at different maize plant growth stages and root locations. Microbiol. Res. 164(4):391-399.

Chan, M.K.Y. 1985. Studies on aphanomyces root rot of peas *(Pisum sativum)* caused by *Aphanomyces euteiches*. PhD Diss. Lincoln College, University of Canterbury.

Chandrashekara, C., J.C. Bhatt, R. Kumar and K.N. Chandrashekara. 2012. Suppressive soils in plant disease management. Eco-Friendly Innovative Approaches in Plant Disease Management, Ed. A. Singh; New Delhi: International Book Distributors. 241-256.

Chandrashekhara, S., B.K. Nanjwade, P.S. Goudanavar, F.V. Manvi, and M.S. Ali. 2010. Isolation and characterization of antibiotic production from soil isolates by fermentation. RJPDFT 2(1): 32-36.

Chatterton, S. 2017. Update on pea root rot in 2016. Lethbridge Research Centre, Agronomy Update. 1-31.

Choi, G.J., J.C. Kim, E.J. Park, Y.H. Choi, K.S. Jang, H.K. Lim, K.Y. Cho, and S.W. Lee. 2006. Biological control activity of two isolates of *Pseudomonas fluorescens* against rice sheath blight. Plant Pathol. J. 22(3): 289-294.

Chowdhury, G., U. Sarkar, S. Pullen, W.R. Wilson, A. Rajapakse, T. Fuchs-Knotts, and K.S. Gates. 2012. DNA strand cleavage by the phenazine di-N-oxide natural product myxin under both aerobic and anaerobic conditions. Chem. Res. Toxicol. 25(1):197-206.

Clezy, G. 2016. Root rot survey results from 2016. Saskatchewan Pulse Growers. 1-3.

Conner R.L., K.F. Chang, S.F. Hwang, T.D. Warkentin, and K.B. McRae. 2013. Assessment of tolerance for reducing yield losses in field pea caused by aphanomyces root rot. Can J Plant Sci. 93(3): 473–82.

de Lima Procópio, R.E., I.R. da Silva, M.K. Martins, J.L. de Azevedo, and J.M. de Araújo. 2012. Antibiotics produced by *Streptomyces*. Braz J Infect Dis. 16(5): 466-471.

de Weert, S., and G. Bloemberg. 2006. Rhizosphere competence and the role of root colonization in biocontrol. In: S. Gnanamanickam editor, Plant-Associated Bacteria. Netherlands: Springer 317–333.

Dessaux, Y., C. Grandclément, and D. Faure. 2016. Engineering the rhizosphere. rends Plant Sci. 21(3): 266-278.

Drechsler, C. 1925. Root-rot of peas in the middle Atlantic states in 1924. Phytopathology, 15(1):4.

Felix, M. V. Perez-Puyana, A. Romero, and A. Guerrero. 2017. Development of thermally processed bioactive pea protein gels: evaluation of mechanical and antioxidant properties. Food Bioprod. process 101: 74-83.

Figuerola, E.L., L.D. Guerrero, S.M. Rosa, L. Simonetti, M.E. Duval, J.A. Galantini, J.C. Bedano, L.G. Wall, and L. Erijman. 2012. Bacterial indicator of agricultural management for soil under no-till crop production. PLoS One. 7(11): 51075.

Francl, L.J. 2001. The Disease Triangle: A plant pathological paradigm revisited. The Plant Health Instructor. DOI: 10.1094/PHI-T-2001-0517-01.

Ganeshan, G. and K.A. Manoj. 2005. *Pseudomonas fluorescens*, a potential bacterial antagonist to control plant diseases. J. Plant Interact. 1(3): 123-134.

Gangneux, C., M.A. Cannesan, M. Bressan, L. Castel, A. Moussart, M. Vicré-Gibouin, A. Driouich, I. Trinsoutrot-Gattin, and K. Laval. 2014. A sensitive assay for rapid detection and quantification of *Aphanomyces euteiches* in soil. Phytopathol. 104(10): 1138-1147.

Gaulin E., A. Bottin, C. Jacquet, and B. Dumas. 2009. *Aphanomyces euteiches* and Legumes. Oomycete Genetics and Genomics. Wiley Online Library, 345-360.

Gaulin, E, C. Jacquet, A. Bottin, and B. Dumas. 2007. Root rot disease of legumes caused by *Aphanomyces euteiches*. Mol. Plant Pathol. 8(5): 539-548.

Gaulin, E., M.A. Madoui, A. Bottin, C. Jacquet, C. Mathé, A. Couloux, P. Wincker, and B. Dumas. 2008. Transcriptome of *Aphanomyces euteiches*: new oomycete putative pathogenicity factors and metabolic pathways. PLoS One. 3(3): 1723.

Gómez, E.R., J. Postma, J.M. Raaijmakers, and I. de Bruijn. 2015. Diversity and activity of *Lysobacter* species from disease suppressive soils. Front. Microb. 6: 1243.

González-Sánchez, M.Á., R.M. Pérez-Jiménez, C. Pliego, C. Ramos, A. De Vicente, and F.M. Cazorla. 2010. Biocontrol bacteria selected by a direct plant protection strategy against avocado white root rot show antagonism as a prevalent trait. J. Appl. Bacteriol.109(1): 65-78.

Gopalakrishnan, S., A. Sathya, R. Vijayabharathi, R.K. Varshney, C.L. Gowda, and L. Krishnamurthy. 2015. Plant growth promoting rhizobia: challenges and opportunities. Biotech. 5(4): 355-377.

Government of Saskatchewan, 2012. Dry pea. http://publications.gov.sk.ca/documents/20/86385-dry%20pea.pdf

Gregory, E., S. Forster, H. Kandel, J. Pasche, M. Wunsch, J. Knodel, and K. Hellevang 2016. NDSU Extension Service, 1-11.

Grimont, F. and P.A.D. Grimont. 1992. The genus *Serratia*. In: A. Balows, H.G. Truper, M. Dworkin, W. Harder and K.H. Schleifer editors. The prokaryotes a handbook on the biology of bacteria: ecophysiology, isolation, identification, applications. Spinger Verlag, New York. 2(3): 2823-2848.

Grünwald, N.J., W. Chen, and R.C. Larsen. 2004. Pea diseases and their management. In: Naqvi S.A.M.H. editor. Diseases of Fruits and Vegetables: Springer Dordrecht 2: 301-331.

Guide for Crop Protection. 2018. Chemical management of weeds, plant disease and insects. 1-651.

Haldar, S. and S. Sengupta. 2015. Plant-microbe cross-talk in the rhizosphere: insight and biotechnological potential. Open Microbiol. J. 9: 1.

Han, S. H., S.J. Lee, J.H. Moon, K.H. Park, K.Y. Yang, B.H. Cho, K.Y. Kim, Y.W. Kim, M.C. Lee, A.J. Anderson, and Y.C. Kim. 2006. GacS-dependent production of 2R,3R-butanediol by Pseudomonas chlororaphis O6 is a major determinant for eliciting systemic resistance against Erwinia carotovora but not against *Pseudomonas syringae* pv. tabaci in tobacco. Mol. Plant Microbe Interact. 19: 924-930.

Hanson, K.G. and M.R. Fernandez. 2002. Evaluation of bacterial strains for control of *Fusarium graminearum* and other cereal pathogens. Semiarid Prairie Agricultural Research Centre (SPARC), Agriculture and Agri-Food Canada, Swift Current, Saskatchewan. 1-4.

Hartmann, M., A. Fliessbach, H.R. Oberholzer, and F. Widmer. 2006. Ranking the magnitude of crop and farming system effects on soil microbial biomass and genetic structure of bacterial communities. FEMS Microbiol. Ecol., 57(3): 378-388.

Harveson, R.M., K.A. Nielsen, and K.M. Eskridge. 2014. Utilizing a preplant soil test for predicting and estimating root rot severity in sugar beet in the central high plains of the United States. Plant Dis. 98(9): 1248-1252.

Hass, D., and G. Defago. 2005. Biological control of soil born pathogens by *fluorescent Pseudomonads*. Nature Rev. Microbiol. 3: 307-319.

Hayward, A.C., N. Fegan, M. Fegan, and G.R. Stirling. 2010. Stenotrophomonas and Lysobacter: ubiquitous plant-associated gamma-proteobacteria of developing significance in applied microbiology. J. Appl. Microbiol. 108(3): 756-770.

Heungens, K. and J.L. Parke. 2001. Post-infection biological control of oomycete pathogens of pea by *Burkholderia cepacia* AMMDR1. Phytopathology 91(4): 383-391.

Heyman, F., B. Lindahl, L. Persson, M. Wikstrom, and J. Stenlid. 2007. Calcium concentrations of soil affect suppressiveness against aphanomyces root rot of pea. Soil Biol. Biochem. 39(9): 2222-9.

Hong, C.E., S.Y. Kwon, and J.M. Park. 2016. Biocontrol activity of *Paenibacillus polymyxa* AC-1 against *Pseudomonas syringae* and its interaction with *Arabidopsis thaliana*. Microbiol. Res. 185: 13-21.

Hossain, S, G. Bergkvist, K. Berglund, R. Glinwood, P. Kabouw, A. Mårtensson, and P. Persson. 2014. Concentration and time dependent effects of isothiocyanates produced from Brassicaceae shoot tissues on the pea root rot pathogen *Aphanomyces euteiches*. J. Agric. Food Chem. 62(20): 4584-91.

Hughes, T.J. and C.R. Grau. 2013. Aphanomyces root rot (common root rot) of legumes. Aphanomyces root rot (common root rot) of legumes. Plant Health Instr. DOI: 10.1094/PHI-I2007-0418-01.

Hwang, S.F., B.D. Gossen, K.F. Chang, G.B. Turnbull, R.J. Howard, and S.F. Blade. 2003. Etiology, impact and control of *Rhizoctonia* seedling blight and root rot of chickpea on the Canadian prairies. Can J Plant Sci. 83(4): 959-67

Islam, M.T., 2008. Disruption of ultrastructure and cytoskeletal network is involved with biocontrol of damping-off pathogen *Aphanomyces cochlioides* by *Lysobacter* sp. strain SB-K88. Biol. Control 46(3): 312-321.

Islam, M.T., Y. Hashidoko, A. Deora, T. Ito, and S. Tahara, 2004. Interactions between rhizoplane bacteria and a phytopathogenic *Peronosporomycete Aphanomyces cochlioides* in relation to the suppression of damping-off disease in sugar beet and spinach. IOBC/ WPRS Bulletin 27 (8): 255-260.

Islam, M.T., Y. Hashidoko, A. Deora, T. Ito, and S. Tahara. 2005. Suppression of damping-off disease in host plants by the rhizoplane bacterium *Lysobacter* sp. strain SB-K88 is linked to plant colonization and antibiosis against soilborne *Peronosporomycetes*. Appl. Environ. Microbiol. 71: 3786-3796.

Islam, T., M. Sakasai, Y. hashidoko, A. Deora, Y. Sakihama, and S. Tahara. 2007. Composition of culture medium influences zoosporogenesis and differentiation of *Aphanomyces cochliodes*. J. Gen. Plant Pathol. 73: 324-329.

Jayaraj, J., T. Parthasarathi, and N.V. Radhakrishnan. 2007. Characterization of a *Pseudomonas fluorescens* strain from tomato rhizosphere and its use for integrated management of tomato damping-off. Biocontrol. Springer 52: 683-702.

Ji, G.H., L.F. Wei, Y.Q. He, Y.P. Wu, and X.H. Bai. 2008. Biological control of rice bacterial blight by Lysobacter antibioticus strain 13-1. Biol. Control. 45(3): 288-296.

Jia, X., Y. Zhao, W. Wang, and Y. He. 2015. Elevated temperature altered photosynthetic products in wheat seedlings and organic compounds and biological activity in rhizosphere soil under cadmium stress. Sci. Rep. 5: 14426.

John, M.W. 2001. Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. 52: 487-511.

Johnson, K.B., V.O. Stockwell, and T.L. Sawyer. 2004. Adaptation of fire blight forecasting to optimize the use of biological controls. Plant Dis. 88: 41-48.

Walton, J.D. 1996. Host-selective toxins: agents of compatibility. The plant cell, 8(10): 1723.

Jones, E.E., A. Stewart, N. Bolstridge, and S. Card. 2009. Use of rifampicin-resistant bacterial biocontrol strains for monitoring survival in soil and colonization of pea seedling roots. N. Z. Plant Prot. 62: 34-40.

Jones, F.R, and C. Drechsler.1925. Root rot of peas in the United States caused by *Aphanomyces euteiches*. J. Agric. Res. 30(4): 293-325.

Jones, F.R., and C. Drechsler. 1925. Root rot of peas in the United States caused by *Aphanomyces euteiches*. J. Agric Res. 30: 293-325.

Jones, F.R., and M.B. Linford. 1925. Pea disease survey in Wisconsin. Madison: Agricultural Experiment Station of the University of Wisconsin.

Kalra, Y.P. and J.S. Bhatti. 2006. Soil testing. In Encyclopedia of Soil Science. Taylor & Francis, New York, New York, USA. 1755-1758.

Kelner, A., 1948. A method for investigating large microbial populations for antibiotic activity. J. Bacteriol. 56(2): 157.

Khabbaz, S.E., and P.A. Abbasi. 2013. Isolation, characterization, and formulation of antagonistic bacteria for the management of seedlings damping-off and root rot disease of cucumber. Can. J. Microbiol. 60(1): 25-33.

Ko, H.S., R.D. Jin, H.B. Krishnan, S.B. Lee, and K.Y. Kim. 2009. Biocontrol ability of *Lysobacter antibioticus* HS124 against *Phytophthora* blight is mediated by the production of 4-hydroxyphenylacetic acid and several lytic enzymes. Curr. Microbiol. 59(6): 608-615.

Köhl, J, J. Postma, P. Nicot, M. Ruocco, and B. Blum. 2011. Stepwise screening of microorganisms for commercial use in biological control of plant-pathogenic fungi and bacteria. Bio Control. 57: 1-12.

Koike, S.T., P. Gladders, and A. Paulus. 2006. Vegetable diseases*:* A colour handbook. CRC Press. 1-167.

Krechel, A., A. Faupel, J. Hallmann, A. Ulrich, and G. Berg. 2002. Potato-associated bacteria and their antagonistic potential towards plant-pathogenic fungi and the plant-parasitic nematode *Meloidogyne incognita* (Kofoid and White) Chitwood. Can. J. Microbiol.48(9):772-786.

Krupinsky, J, K. Bailey, M. McMullen, B. Gossen, and T.T. Kelly. 2002. Managing plant disease risk in diversified cropping systems. Agron J 94(2): 198-209.

Kumar, P., V.K. Gupta, A.K. Tiwari, and M. Kamle. 2016. Current Trends in Plant Disease Diagnostics and Management Practices. Springer.

Kumar, S, G. Stecher, and K. Tamura. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33: 1870-1874.

Kurze, S., H. Bahl, R. Dahl, and G. Berg, 2001. Biological control of fungal strawberry diseases by *Serratia plymuthica* HRO-C48. Plant Dis., 85(5): 529-534.

Lavaud, C., A. Lesne, C. Piriou, G. Le Roy, G. Boutet, A. Moussart, C. Poncet, R. Delourme, A. Baranger, and M.L. Pilet-Nayel. 2015. Validation of QTL for resistance to *Aphanomyces euteiches* in different pea genetic backgrounds using near-isogenic lines. Theor. Appl. Genet. 128(11):2273-2288.

Law, J.W.F., H.L. Ser, T.M. Khan, L.H. Chuah, P. Pusparajah, K.G. Chan, B.H. Goh, and H.L. Lee. 2017. The potential of Streptomyces as biocontrol agents against the rice blast fungus, Magnaporthe oryzae (Pyricularia oryzae). Front. Microb. 8: 3.

Lee, Y.S. Naning, K.W. Nguyen, X.H. Kim, S.B. Moon, J.H. and K.Y. Kim. 2014. Ovicidal activity of lactic acid produced by *Lysobacter capsici* YS1215 on eggs of root-knot nematode, *Meloidogyne incognita*. J. Microbiol. Biotechnol. 24(11): 1510-1515.

Lejbølle, K.B. 2004. Molecular and ecological factors affecting survival and activities of the biocontrol agent *Pseudomonas fluorescens* CHA0 Ph.D. diss., National Environmental Research Institute.

Liu, X., J. Jia, S. Atkinson, M. Cámara, K. Gao, H. Li, and J. Cao. 2010. Biocontrol potential of an endophytic *Serratia* sp. G3 and its mode of action. World J. Microbiol. Biotechnol. 26(8): 1465-1471.

Lozano, G.L., J. Holt, J. Ravel, D.A. Rasko, M.G. Thomas, and J. Handelsman. 2016. Draft genome sequence of biocontrol agent *Bacillus cereus* UW85. Genome announcements, 4(5): 00910-16.

Maheshwari, D.K. editor. 2017. Endophytes: Biology and Biotechnology. Springer International Publishing.

Mauchline, T.H., and J.G. Malone. 2017. Life in earth–the root microbiome to the rescue? Curr. Opin. Microbiol. 37: 23-28.

McGee, R.J., Coyne, C.J., Pilet-Nayel, M.L., Moussart, A., Tivoli, B., Baranger, A., Hamon, C., Vandemark, G. and McPhee, K. 2012. Registration of pea germplasm lines partially resistant to aphanomyces root rot for breeding fresh or freezer pea and dry pea types. J. Plant Regist. 6(2):203-207.

McKay, K., B.G. Schatz, and G. Endres. 2003. Field pea production. NDSU Extension Service. 1-8.

Montesinos, E. 2003. Plant-associated microorganisms: a view from the scope of microbiology. 221-223.

Mota, M.S., C.B. Gomes, I.R. Souza Júnior, and A.B. Moura. 2017. Bacterial selection for biological control of plant disease: criterion determination and validation. Braz. J. Microbiol. 48(1): 62-70.

Moussart, A, E. Lemarchand, and B. Tivoli. 2006. Description, validation, possible uses of a soil infectivity test for *Aphanomyces euteiches*. AFPP, In 8th international conference on plant diseases, Tours, Dec. 5-6.

Mutka, A.M., and R.S. Bart. 2015. Image-based phenotyping of plant disease symptoms. Front. Plant Sci.5: 734.

Naqvi, S.A. 2007. Diseases of fruits and vegetables: volume II: diagnosis and management (vol. 2). Springer Science and Business Media.

Njoka, J.G. 2008. Effects of feeding Iowa-grown field peas on finishing pig performance. Iowa State University Capstones 1-65.

Ohike, T., M. Maeda, T. Matsukawa, M. Okanami, S.I. Kajiyama, and T. Ano. 2017. *In vitro* and *in vivo* assay for assessment of the biological control potential of *Streptomyces* sp. KT. J. Plant Stud.7(1): 10.

Oyarzun, P.J. 1994. Root rot of peas in the Netherlands; fungal pathogens, inoculum potential and soil receptivity. Ph.D. diss., Wageningen, the Netherlands.

Pal, K.K. and McSpadden G.B.M. 2006. Biological control of plant pathogens. Plant Health Instr. http://dx.doi.org/10.1094/PHIA-2006- 1117-02

Panpatte, D.G., Y.K. Jhala, H.N. Shelat, and R.V. Vyas. 2016. *Pseudomonas fluorescens*: a promising biocontrol agent and PGPR for sustainable agriculture. In Microbial inoculants in sustainable agricultural productivity. Springer, New Delhi 257-270.

Papavizas, G.C., and W.A. Ayers 1974. *Aphanomyces* species and their root diseases in pea and sugar beet: a review Report. Washington, DC: Agricultural Research Service, US Department of Agriculture; Sep. Report No.:1485.

Park, J.H., R. Kim, Z. Aslam, C.O. Jeon, and Y.R. Chung. 2008. *Lysobacter capsici* sp. nov., with antimicrobial activity, isolated from the rhizosphere of pepper, and emended description of the genus *Lysobacter*. Int. J. Syst. Evol. Microbiol. 58(2): 387-392.

Parke, J. L., R.E. Rand, A.E. Joy, and E.B. King. 1991. Biological control of *Pythium* damping-off and aphanomyces root rot of peas by application of *Pseudomonas cepacia* or *P. fluorescens* to seed. Plant Dis. 75: 987-992.

Paul, E.A. 2014. *Soil microbiology, ecology and biochemistry*. Academic press Nov. 14.

Pereg, L., and M. McMillan. 2015. Scoping the potential uses of beneficial microorganisms for increasing productivity in cotton cropping systems. Soil Biol. Biochem.80: 349-358.

Persson, L., and S. Olsson. 2000. Abiotic characteristics of soils suppressive to aphanomyces root rot. *Soil* Soil Biol. Biochem. 32(8-9): 1141-1150.

Peterson, E. A., D.C. Gillespie, and F.D. Cook. 1966. A wide spectrum antibiotic produced by a species of Sorangium Can. J. Microbiol. 12, 221-230.

Prashar, P., N. Kapoor, and S. Sachdeva. 2014. Rhizosphere: its structure, bacterial diversity and significance. Rev. Environ. Sci. Bio/Technol. 13(1): 63-77.

Pusey, P.L. 2002. Biological control agents for fire blight of apple compared under conditions limiting natural dispersal. Plant Dis. 86: 639-644.

Pusey, P.L., V.O. Stockwell, and D.R. Rudell. 2008. Antibiosis and acidification by *Pantoea agglomerans* strain E325 may contribute to suppression of *Erwinia amylovora*. Phytopathology 98(10): 1136-1143.

Qian, G., Y. Wang, D. Qian, J. Fan, B. Hu, and F. Liu. 2012. Selection of available suicide vectors for gene mutagenesis using chiA (a chitinase encoding gene) as a new reporter and primary functional analysis of chiA in Lysobacter enzymogenes strain OH11. World J. Microbiol Biotechnol. 28(2):549-557.

Reichenbach, H. 2006. The Genus *Lysobacter*, In: the prokaryotes Dworkin M., S. Falkow, E. Rosenberg K.H. Schleifer, E. Stackebrandt editors. New York, NY: Springer 939-957.

Rezzonico, F., T.H.M. Smits, E. Montesinos, J.E. Frey, and B. Duffy. 2009. Genotypic comparison of *Pantoea agglomerans* plant and clinical strains. BMC Microbiol. 9(1): 204.

Rousk, J., P.C. Brookes, and E. Bååth. 2009. Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. Appl. Environ. Microbiol.75(6): 1589-1596.

Rowell, D. 1994. Soil Science. Methods and Applications. Longman, Harlow 350.

Ruocco M., S. Woo, F. Vinale, S. Lanzuise, and M. Lorito. 2011. "Identified difficulties and conditions for field success of biocontrol. 2. Technical aspects: factors of efficacy," in classical and augmentative biological control against diseases and pests: Critical status analysis and review of factors influencing their success, ed. Nicot P. C. 45–57.

Salaheddin, K., V. Valluvaparidasan, D. Ladhalakshmi, and R. Velazhahan. 2010. Management of bacterial blight of cotton using a mixture of *Pseudomonas fluorescens* and *Bacillus subtilis*. Plant Prot. Sci. 46: 41-50.

Santoyo, G., M.D.C. Orozco-Mosqueda, and M. Govindappa. 2012. Mechanisms of biocontrol and plant growth-promoting activity in soil bacterial species of *Bacillus* and *Pseudomonas*: a review. Biocontrol Sci. Technol. 22: 855-972.

Saskatchewan pulse grower. 2017. Root rot in peas and lentils in western Canada. https://saskpulse.com/files/technical_documents/170418_Root_Rot_Brochure_v7_LR1.pdf

Saskatchewan Pulse Growers. 2016, Pulse Market Report. https://saskpulse.com/files/report/151223_PMR_January_2016_OUTPUT.pdf

Sauvage, H., A. Moussart, F. Bois, B. Tivoli, S. Barray, and K. Laval. 2007. Development of a molecular method to detect and quantify *Aphanomyces euteiches* in soil. FEMS Microbiol. Lett. 273(1): 64-69.

Scher, F.M., J.W. Kloepper, C. Singleton, I. Zaleska, and M. Laliberte. 1988. Colonization of soybean roots by *Pseudomonas* and *Serratia* species: Relationship to bacterial motility, chemotaxis, and generation time. Phytopathology 78: 1055-1059.

Schjønning, P., J.L. Jensen, S. Bruun, L.S. Jensen, B.T. Christensen, L.J. Munkholm, M. Oelofse, S. Baby, and L. Knudsen. 2018. The role of soil organic matter for maintaining crop yields: Evidence for a renewed conceptual basis. Adv. Agron. 150: 35-79.

Segarra, G., G. Puopolo, E. Porcel-Rodríguez, O. Giovannini, and I. Pertot. 2015. Monitoring *Lysobacter capsici* AZ78 using strain specific qPCR reveals the importance of the formulation for its survival in vineyards. FEMS Microbiol. Lett. 363(3): 243.

Sharma, M., P. Dangi, and M. Choudhary. 2014. *Actinomycetes*: source, identification, and their applications. Int J Curr Microbiol App Sci. 3(2): 801-832.

Silva, G.A.D., and E.A.D. Almeida. 2006. Production of yellow-green fluorescent pigment by *Pseudomonas fluorescens*. Braz. Arch. Biol. Technol. 49(3): 411-419.

Singh, D.P., H.B. Singh, and R. Prabha editors. 2016. Microbial inoculants in sustainable agricultural productivity. New York, NY: Springer.

Slinkard, A.E., C. van Kessel, D.E. Feindel, S.T. Aii-Khan, and R. Park. 1994. Addressing farmers' constraints through on-farm research: peas in western Canada. In: Expanding the production and use of cool season food legumes. Muehlbauer. F.J., and w.j. Kaiser, editors. New York: Kluwer Academic Publisher 877–89.

Slusarenko, K.L. 2004. A study of *Aphanomyces euteiches* Drechs. root rot of field pea (Pisum sativum L.) in Manitoba.

Souza, R.D., A. Ambrosini, and L.M. Passaglia. 2015. Plant growth-promoting bacteria as inoculants in agricultural soils. Genet. Mol. Biol. 38(4):401-419.

Spies, J.M. 2008. The effect of field pea (*Pisum sativum* L.) basal branching on optimal plant density and crop competitiveness. PhD Diss. University of Saskatchewan, Saskatoon.

Spies, J.M., T.D. Warkentin, and S.J. Shirtliffe. 2011. Variation in field pea (*Pisum sativum*) cultivars for basal branching and weed competition. Weed Sci. 59(2): 218-223.

Srividya, A.R., G.S. Saritha, and B. Suresh. 2008. Study of the soil isolates for antimicrobial activity Indian J. Pharm. Sci. 70(6): 812

Srividya, S., A. Thapa, D.V. Bhat, K. Golmei, and N. Dey, 2012. *Streptomyces* sp. 9p as effective biocontrol against chilli soilborne fungal phytopathogens. Eur. J. Exp. Biol, 2(1): 163-173.

Statistics Canada. 2018. https://www150.statcan.gc.ca/n1/en/daily-quotidien/180629/dq180629b-eng.pdf?st=kHT8DbMO

Stockwell, V.O., K.B. Johnson, D. Sugar, J.E. Loper. 2010. Control of fire blight by *Pseudomonas fluorescens* A506 and *Pantoea vagans* C9-1 applied as single strains and mixed inocula. Phytopathology 100(12): 1330-1339.

Chatterton S, S. Banniza, R. Bowness, M. Harding, B. Gossen and D. McLaren. 2017. Root rot complex in western Canada where are we at with *Fusarium*, *Aphanomyces*, and *Phytophthora*. Agriculture and Agri-Food Canada, 1-43.

Tripathi, J., A.K. Singh, and P. Tiwari. 2013. Studies on heterotrophic bacteria with special reference to *Azospirillum* from rhizosphere and root of different crops. Afr. J. Agric. Res. 8(26): 3436-3443.

Validov, S., F. Kamilova, S. Qi, D. Stephan, J.J. Wang, N. Makarova, and B. Lugtenberg. 2007. Selection of bacteria able to control *Fusarium oxysporum* f. sp. radicis-lycopersici in stonewool substrate. J Appl Microbiol. 102(2): 461-471.

Vandemark, G.J., and L.D. Porter. 2010. 878001. First report of lentil root rot caused by *Aphanomyces euteiches* in Idaho. Plant dis.94(4): 480-480.

Vanneste, JL, D.C. Cornish, J. Yu, and M.D. Voyle. 2002. P10c: a new biological control agent for control of fire blight which can be sprayed or distributed using honey bees. Acta Hortic. 590: 231-235.

Vasilyeva, N.V., N.A. Shishkova, L.I. Marinin, L.A. Ledova, I.M. Tsfasman, T.A. Muranova, O.A. Stepnaya, and I.S. Kulaev. 2014. Lytic peptidase L5 of *Lysobacter* sp. XL1 with broad antimicrobial spectrum. J. Mol. Microbiol. Biotechnol. 24(1): 59-66.

Vasudevan, P., S. Kavitha, V.B. Priyadarisini, L. Babujee, and S.S. Gnanamanickam. 2002. Biological control of rice diseases. In: biological control of crop diseases. Gnanamanickam S.S., editor. New York, NY: Marcel Dekker Inc.

Vincent, J.M. 1970. A manual for the practical study of root-nodule bacteria. Blackwell Scientific: Oxford, England.

Vittaladevaram, V., K. Kuruti, B. Gandu, and U. Sudheer. 2017. Journal of chemical and pharmaceutical research. J. Chem. Pharm. Res. 9(6): 80-85.

Vurukonda, S.S.K.P., D. Giovanardi, and E. Stefani. 2018. Plant growth promoting and biocontrol activity of *Streptomyces* spp. as endophytes. Int. J. Mol. Sci., 19(4): 952.

Wakelin, S.A., M. Walter, M. Jaspers, and A. Stewart. 2002. Biological control of *Aphanomyces euteiches* root-rot of pea with spore-forming bacteria. Australas. Plant Pathol. 31(4): 401-407.

Waksman, S.A. 1945. Microbial antagonisms and antibiotic substances. Commonwealth Fund.; New York.

Wang, D, ands D.W. Anderson. 1998. Direct measurement of organic carbon content in soils by the Leco CR-12 carbon analyzer. Commun Soil Sci Plant Anal 29: 15-21.

Wang, H., S.F. Hwang, K.F. Chang, G.D. Turnbull, and R.J. Howard. 2003. Suppression of important pea diseases by bacterial antagonists. BioControl 48(4): 447-460.

Wang, S., P. Sharp, and L. Copeland. 2011. Structural and functional properties of starches from field peas. Food Chem.126(4): 1546-1552.

Weissmann, R. and B. Gerhardson. 2001. Selective plant growth suppression by shoot application of soil bacteria. Plant and soil 234(2): 159-170.

Weller, D.M. 2007. *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. Phytopathology, 97(2): 250-256.

Willsey, T.L., S. Chatterton, M. Heynen, and A. Erickson. 2018. Detection of interactions between the pea root rot pathogens *Aphanomyces euteiches* and *fusarium* spp. using a multiplex qPCR assay. Plant Pathol. 67: 1912-1923.

Wright, S.A., C.H. Zumoff, L. Schneider, and S.V. Beer. 2001. *Pantoea agglomerans* strain EH318 produces two antibiotics that inhibit *Erwinia amylovorain* vitro. Appl. Environ. Microbiol. 67(1): 284-292.

Wu, L.F. 2018. Occurrence and management of root rot of field pea cause by *Aphanomyces euteiches* [dissertation] University of Alberta, Edmonton.

Wu, L., K.F. Chang, R.L. Conner, S. Strelkov, R. Fredua-Agyeman, S.F. Hwang, and D. Feindel, 2018. *Aphanomyces euteiches*: A threat to Canadian field pea production. *Engineering*.

Xie, Y., S. Wright, Y. Shen, and L. Du. 2012. Bioactive natural products from *Lysobacter*. Nat Prod Rep. 29(11): 1277-87.

Xu, G.W., and D.C. Gross. 1986. Field evaluations of the interactions among *fluorescent pseudomonads*. *Erwinia carotovora*. 423-430.

Xue, A.G. 2003. Efficacy of *Clonostachys rosea* strain ACM941 and fungicide seed treatments for controlling the root rot complex of field pea. Can. J. Plant Sci. 83(3): 519-24.

Yadav, A.N., P. Verma, B. Singh, and V.S. Chauahan. 2017. Plant growth promoting bacteria: biodiversity and multifunctional attributes for sustainable agriculture. Adv Biotechnol Microbiol. 5(5): 1-16.

Yang, J.H., H.X. Liu H. G.M. Zhu, Y.L. Pan, L.P. Xu, and H.J. Guo. 2008. Diversity analysis of antagonists from rice-associated bacteria and their application in biocontrol of rice diseases. J. Appl. Microbiol. 104(1): 91-104.

Ye, W., X. Liu, S. Lin, J. Tan, J. Pan, D. Li, and H. Yang. 2009. The vertical distribution of bacterial and archaeal communities in the water and sediment of Lake Taihu. FEMS Microbiol. Ecol. 70(2): 263-276.

Yuen, G.Y., J.R. Steadman, D.T. Lindgren, D. Schaff, and C. Jochum. 2001. Bean rust biological control using bacterial agents1. Crop Prot. 20(5): 395-402.

Zeigler, D.R., and J.B. Perkins. 2015. 27 The Genus Bacillus. Pract. Handb. Microbiol. 429.

Zhang, Z., G.Y. Yuen, G. Sarath, and A.R. Penheiter. 2001. Chitinases from the plant disease biocontrol agent, *Stenotrophomonas maltophilia* C3. Phytopathology 91: 204–211.

Zohary, D., and M. Hopf. 2002. Domestication of plants in the old world: The origin and spread of cultivated plants in west Asia, Europe and the Nile Valley. Third Edition. Oxford University Press Inc. New York.

APPENDIX A: DATA FOR BIOCONTROL ASSESSMENT IN VERMICULITE

Table A. 1 Disease score data for initial growth chamber screening of bacterial isolates against aphanomyces root rot in field pea grown in vermiculite; set 1.

Antagonistic bacterial isolates	Disease level				Mean comparison
	R1	R2	R3	R4	
Negative control (Control C) †	0	0	0	0	0.00[d]
DR1-2 (*Serratia plymuthica*)	0	0	0	0	0.00[d]
H4-5 (*Pseudomonas* spp)	0	0	0	0	0.00[d]
Hf-L7 (*Paenibacillus* spp)	0	1	0	0	0.25[cd]
W4-3 (*Paenibacillus polymyxa*)	0	0	1	1	0.25[cd]
PK1-11 (*Bacillus atrophaeus*)	0	0	0	1	0.25[cd]
Hf-L2 (*Pseudomonas* spp)	1	1	0	0	0.50[cbd]
Ler3-4 (*Pseudomonas syringae*)	0	1	1	0	0.50[cbd]
Ler4-2 (*Serratia plymuthica*)	1	1	1	0	0.75[cb]
NB4-2 (*Pseudomonas fluorescens*)	1	1	1	1	1.00[b]
NB2-1 (*Brevibacillus laterosporus*)	2	2	2	1	1.75[a]
Positive control (Control B)	2	2	2	2	2.00[a]
					LSD = 0.72

† Negative control: uninoculated pea plants; Positive control: pea plants inoculated with *A. euteiches* zoospore. Plants inoculated with suspensions of each antagonistic bacterial isolates only (Control A) did not manifested visually detectably phytotoxic or phytopathogenic symptoms derived from the antagonistic bacteria. Means across all column followed by same letters are not significantly different at $\alpha = 0.05$ using Fisher Least Significance Difference Test. The data were collected on a 0-4 disease level scale. Each treatment was laid out in a complete randomized design in four replicates (R).

Table A.2 Disease score data for initial growth chamber screening of bacterial isolates against aphanomyces root rot in field pea grown in vermiculite; set 2.

Antagonistic bacterial isolates	Disease level				Mean comparison
	R1	R2	R3	R4	
Negative control (Control C) †	0	0	0	0	0.00[d]
(DR1-3) *Pseudomonas spp*	0	0	0	0	0.00[d]
(Ler3-1) *Bacillus stratosphericus*	0	0	0	0	0.00[d]
(CB3-1) *Pseudomonas fluorescens*	1	1	0	0	0.50[cbd]
(Hf-L4) *Pseudomonas* spp	0	1	0	1	0.50[cbd]
(Ler4-1) *Serratia plymuthica*	1	1	0	0	0.50[cbd]
E3-1 (*Streptomyces sp.*)	1	0	1	0	0.50[cbd]
H2-7 (*Pseudomonas sp.*)	1	0	0	1	0.50[cbd]
(PSV1-15) *Paenibacillus pabuli*	1	0	1	1	0.75[cb]
(PCB1-13) *Pseudomonas mucidolens*	1	1	1	1	1.00[b]
(DR3-1) *Bacillus thuringiensis*	2	2	2	2	2.00[a]
Positive control (Control B)	2	2	2	2	2.00[a]
					LSD = 0.60

† Negative control: uninoculated pea plants; Positive control: pea plants inoculated with *A. euteiches* zoospore. Plants inoculated with suspensions of each antagonistic bacterial isolates only (Control A) did not manifested visually detectably phytotoxic or phytopathogenic symptoms derived from the antagonistic bacteria Means across all column followed by same letters are not significantly different at $\alpha = 0.05$ using Fisher Least Significance Difference Test. The data were collected on a 0-4 disease level scale. Each treatment was laid out in a complete randomized design in four replicates (R).

Table A.3 Disease score data for initial growth chamber screening of bacterial isolates against aphanomyces root rot in field pea grown in vermiculite; set 3.

Antagonistic bacterial isolates	Disease level				Mean comparison
	R1	R2	R3	R4	
Negative control (Control C) †	0	0	0	0	0.00[c]
DR4-4 (*Bacillus thuringiensis*)	0	0	0	0	0.00[c]
MB-H5 (*Bacillus stratosphericus*)	0	0	0	0	0.00[c]
NB4-3 (*Streptomyces* spp)	1	0	0	0	0.25[c]
DR1-1 (*Pseudomonas* spp)	1	1	0	0	0.50[cb]
Hf-L6 (*Pseudomonas* spp)	1	0	1	0	0.50[cb]
PSV1-7 (*Pantoea agglomerans*)	0	0	1	1	0.50[cb]
CB1-11 (*Pseudomonas* spp)	1	1	1	1	1.00[b]
PCB1-15 (*Pseudomonas mucidolens*)	1	1	1	1	1.00[b]
Hf-L1 (*Pseudomonas* spp)	2	2	2	2	2.00[a]
PSV1-9 (*Rhizobium lemnae*)	2	2	2	2	2.00[a]
Positive control (Control B)	2	2	2	2	2.00[a]
					LSD = 0.61

† Negative control: uninoculated pea plants; Positive control: pea plants inoculated with *A. euteiches* zoospore. Plants inoculated with suspensions of each antagonistic bacterial isolates only (Control A) did not manifested visually detectably phytotoxic or phytopathogenic symptoms derived from the antagonistic bacteria Means across all column followed by same letters are not significantly different at $\alpha = 0.05$ using Fisher Least Significance Difference Test. The data were collected on a 0-4 disease level scale. Each treatment was laid out in a complete randomized design in four replicates (R).

Table A.4 Disease score data for initial growth chamber screening of bacterial isolates against aphanomyces root rot in field pea grown in vermiculite; set 4.

Antagonistic bacterial isolates	Disease level				Mean comparison
	R1	R2	R3	R4	
Negative control (Control C) †	0	0	0	0	0.00^{e}
H2-1 (*Bacillus stratosphericus*)	0	0	0	0	0.00^{e}
PCV1-13 (*Rhizobium lemnae*)	0	0	0	0	0.00^{e}
H2-5 (*Paenibacillus polymyxa*)	0	0	0	0	0.00^{e}
O1-2 (*Bacillus* spp)	1	0	0	0	0.25^{de}
DR3-4 (*Bacillus stratosphericus*)	0	0	1	1	0.50^{dce}
Ler1-1 (*Pantoea agglomerans*)	1	0	1	0	0.50^{dce}
CB1-3 (*Lysobacter antibioticus*)	0	1	1	1	0.75^{dc}
Hf-L5 (*Pseudomonas* spp)	1	1	1	1	1.00^{bc}
W2-4 (*Bacillus thuringiensis*)	1	1	1	1	1.00^{bc}
W4-9 (*Bacillus simplex*)	2	1	2	1	1.50^{ba}
Positive control (Control B)	2	2	2	2	2.00^{a}
					LSD = 0.70

† Negative control: uninoculated pea plants; Positive control: pea plants inoculated with *A. euteiches* zoospore. Plants inoculated with suspensions of each antagonistic bacterial isolates only (Control A) did not manifested visually detectably phytotoxic or phytopathogenic symptoms derived from the antagonistic bacteria Means across all column followed by same letters are not significantly different at $\alpha = 0.05$ using Fisher Least Significance Difference Test. The data were collected on a 0-4 disease level scale. Each treatment was laid out in a complete randomized design in four replicates (R).

Table A.5 Disease score data for initial growth chamber screening of bacterial isolates against aphanomyces root rot in field pea grown in vermiculite; set 5.

Antagonistic bacterial isolates	Disease level				Mean comparison
	R1	R2	R3	R4	
Negative-control (Control C) †	0	0	0	0	0.00^{c}
K-Be-H3 (*Lysobacter gummosus*)	0	0	1	0	0.25^{cb}
Ler3-3 (*Streptomyces lavendulae*)	1	0	0	1	0.50^{cb}
K-CB1-1 (Streptomyces paradoxus)	0	1	1	1	0.75^{cb}
K-Hf-L9 (*Pseudomonas fluorescens*)	1	1	1	1	1.00^{b}
Positive control (Control B)	2	2	2	2	2.00^{a}
K-CB2-4 (*Lysobacter antibioticus*)	3	2	2	2	2.25^{a}
K-Hf-H2 (*Lysobacter capsici*)	3	3	2	2	2.50^{a}
K-CB2-6 (*Bacillus-cereus*)	2	3	2	3	2.50^{a}
					LSD = 0.85

† Negative control: uninoculated pea plants; Positive control: pea plants inoculated with *A. euteiches* zoospore. Plants inoculated with suspensions of each antagonistic bacterial isolates only (Control A) did not manifested visually detectably phytotoxic or phytopathogenic symptoms derived from the antagonistic bacteria Means across all column followed by same letters are not significantly different at $\alpha = 0.05$ using Fisher Least Significance Difference Test. The data were collected on a 0-4 disease level scale. Each treatment was laid out in a complete randomized design in four replicates (R).

APPENDIX B: DATA FOR BIOCONTROL ASSESSMENT IN SOIL

Table B.1 Growth chamber biocontrol assessment of bacterial isolates against aphanomyces root rot disease in field pea grown in soil; set 1.

Antagonistic bacterial isolates	Disease level				Mean comparison
	R1	R2	R3	R4	
Negative control (Control C) †	0	0	0	1	0.25[f]
K-Hf- L9 (*Pseudomonas fluorescens*)	1	1	1	1	1.00[c]
PSV1-7 (*Pantoea agglomerans*)	1	1	1	1	1.00[c]
H2-5 (*Paenibacillus polymyxa*)	1	2	2	2	1.75[b]
K-CB2-6 (*Bacillus cereus*)	2	2	1	2	1.75[b]
O1-2 (*Bacillus* spp)	2	1	2	2	1.75[b]
DR1-2 (*Serratia plymuthica*)	2	2	2	2	2.00[b]
K-Be- H3 (*Lysobacter gummosus*)	2	2	2	2	2.00[b]
PCV1-13 (*Rhizobium lemnae*)	2	2	2	2	2.00[b]
CB3-1 (*Pseudomonas fluorescens*)	3	3	3	3	3.00[a]
Ler3-3 (*Streptomyces lavendulae*)	3	3	3	3	3.00[a]
Positive control (Control B)	3	3	3	3	3.00[a]
					LSD = 0.46

† Negative control: uninoculated pea plants; Positive control: pea plants inoculated with *A. euteiches* zoospore. Plants inoculated with suspensions of each antagonistic bacterial isolates only (Control A) did not manifested visually detectably phytotoxic or phytopathogenic symptoms derived from the antagonistic bacteria Means across all column followed by same letters are not significantly different at $\alpha = 0.05$ using Fisher Least Significance Difference Test. The data were collected on a 0-4 disease level scale. Each treatment was laid out in a complete randomized design in four replicates (R).

Table B.2 Growth chamber biocontrol assessment of bacterial isolates against aphanomyces root rot disease in field pea grown in soil; set 1.

Antagonistic bacterial isolates	Disease level				Mean comparison
	R1	R2	R3	R4	
Negative control (Control C) †	0	0	1	0	0.25[d]
(K-Hf-H2) (*Lysobacter capsici*)	0	0	1	1	0.50[d]
Hf-L4 (*Pseudomonas sp.*)	1	2	2	1	1.50[c]
PK1-11 (*Bacillus atrophaeus*)	2	2	2	1	1.75[c]
NB4-3 (*Streptomyces sp.*)	2	2	1	2	1.75[c]
K-CB1-1 (*Streptomyces paradoxus*)	1	2	2	2	1.75[c]
MB-H5 (*Bacillus stratosphericus*)	2	2	2	2	2.00[bc]
DR4-4 (*Bacillus-thuringiensis*)	2	2	2	2	2.00[bc]
H4-5 (*Pseudomonas sp.*)	2	3	2	3	2.50[ba]
K-CB2-4 (*Lysobacter antibioticus*)	3	3	3	3	3.00[a]
Ler3-4 (*Pseudomonas-syringae*)	3	3	3	3	3.00[a]
Positive control (Control B)	3	3	3	3	3.00[a]
					LSD = 0.56

† Negative control: uninoculated pea plants; Positive control: pea plants inoculated with *A. euteiches* zoospore. Plants inoculated with suspensions of each antagonistic bacterial isolates only (Control A) did not manifested visually detectably phytotoxic or phytopathogenic symptoms derived from the antagonistic bacteria Means across all column followed by same letters are not significantly different at $\alpha = 0.05$ using Fisher Least Significance Difference Test. The data were collected on a 0-4 disease level scale. Each treatment was laid out in a complete randomized design in four replicates (R).

APPENDIX C: ANOVA OF THE BIOCONTROL ASSESSMENT IN VERMICULITE

Table C. 1 One-way analysis of variance of aphanomyces root rot development scores in field pea as influenced by biocontrol efficacy of antagonistic bacterial isolates; set 1.

Source of variation	DF	SS	MS	F	P
Between groups	11	19.67	1.79	7.15	<.0001
With in groups	36	9.00	0.25		
Total	47	28.67			

Table C. 2 One-way analysis of variance of aphanomyces root rot development scores in field pea as influenced by biocontrol efficacy of antagonistic bacterial isolates; set 2.

Source of variation	DF	SS	MS	F	P
Between groups	11	18.73	1.79	9.81	<.0001
With in groups	36	6.25	0.17		
Total	47	24.97			

Table C. 3 One-way analysis of variance of aphanomyces root rot development scores in field pea as influenced by biocontrol efficacy of antagonistic bacterial isolates; set 3.

Source of variation	DF	SS	MS	F	P
Between groups	11	25.41	2.3	12.8	<.0001
With in groups	36	6.5	0.18		
Total	47	31.9			

Table C. 4 One-way analysis of variance of aphanomyces root rot development scores in field pea as influenced by biocontrol efficacy of antagonistic bacterial isolates; set 4.

Source of variation	DF	SS	MS	F	P
Between groups	11	18.75	1.7	7.22	<.0001
With in groups	36	8.5	0.24		
Total	47	27.25			

Table C. 5 One-way analysis of variance of aphanomyces root rot development scores in field pea as influenced by biocontrol efficacy of antagonistic bacterial isolates; set 5.

Source of variation	DF	SS	MS	F	P
Between groups	8	32.38	4.04	11.82	<.0001
With in groups	27	9.25	0.34		
total	35	41.63			

APPENDIX D: ANOVA OF THE BIOCONTROL ASSESSMENT IN SOIL

Table D. 1 One-way analysis of variance of aphanomyces root rot development scores in field pea as influenced by biocontrol efficacy of antagonistic bacterial isolates; set 1.

Source of variation	DF	SS	MS	F	P
Between groups	11	30.23	2.75	26.38	<.0001
With in groups	36	3.75	0.1		
Total	47	33.98			

Table D. 2 One-way analysis of variance of aphanomyces root rot development scores in field pea as influenced by biocontrol efficacy of antagonistic bacterial isolates; set 2.

	Disease score				
Source of variation	DF	SS	MS	F	P
Between groups	11	35.67	3.24	21.22	<.0001
With in groups	36	5.50	0.15		
Total	47	41.17			

APPENDIX E: IDENTIFICATION OF THE CANDIDATE BIOCONTROL AGENTS

K-Hf-L9 (*Pseudomonas fluorescens*) nucleotide sequence from primers 27F/1492R

NNNNNNNNNNNNctggcgNNNNccNNNNNatgcaagtcgagcggtagagagaagcttgcttctcttgagagcggcg
gacgggtgagtaatgcctaggaatctgcctggtagtgggggataacgttcggaaacgaacgctaataccgcatacgtcctacgggagaaa
gcaggggaccttcgggccttgcgctatcagatgagcctaggtcggattagctagttggtgaggtaatggctcaccaaggcgacgatccgta
actggtctgagaggatgatcagtcacactggaactgagacacggtccagactcctacgggaggcagcagtggggaatattggacaatgg
gcgaaagcctgatccagccatgccgcgtgtgtgaagaaggtcttcggattgtaaagcactttaagttgggaggaagggcagttacctaatac
gtgattgttttgacgttaccgacagaataagcaccggctaactctgtgccagcagccgcggtaatacagagggtgcaagcgttaatcggaat
tactgggcgtaaagcgcgcgtaggtggtttgttaagttggatgtgaaatccccgggctcaacctgggaactgcattcaaaactgactgacta
gagtatggtagagggtggtggaatttcctgtgtagcggtgaaatgcgtagatataggaaggaacaccagtggcgaaggcgaccacctgga
ctaatactgacactgaggtgcgaaagcgtggggagcaaacaggattagataccctggtagtccacgccgtaaacgatgtcaactagccgtt
ggaagccttgagcttttagtggcgcagctaacgcattaagttgaccgcctggggagtacggccgcaaggttaaaactcaaatgaattgacg
ggggcccgcacaagcggtggagcatgtggtttaattcgaagcaacgcgaagaaccttaccaggccttgacatccaatgaactttctagaga
tagattggtgccttcgggaacattgagacaggtgctgcatggctgtcgtcagctcgtgtcgtgagatgttgggttaagtcccgtaacgagcgc
aacccttgtccttagttaccagcacgtaatggtgggcactctaaggagactgccggtgacaaaccggaggaaggtggggatgacgtcaag
tcatcatggcccttacggcctgggctacacacgtgctacaatggtcggtacagagggttgccaagccgcgaggtggagctaatcccataaa
accgatcgtagtccggatcgcagtctgcaactcgactgcgtgaagtcggaatcgctagtaatcgcgaatcagaatgtcgcggtgaatacgtt
cccgggccttgtacacaccgcccgtcacaccatgggagtgggttgcaccagaagtagctagtctaaccttcgggggggacggNNNNN
NNggtgtgatcatNaNNNNNNNNNNN

PSV1-7 (*Pantoea agglomerans*) nucleotide sequence from primers EUB 338/EUB518

tggNNNttcNggNttcttctgcNgnacgtcatccgacagagttattaaccccgtccgccttccctccccgctgaaagtactttacaaccc
gaaggccttccttccatacacgcggcatggctgcatcaggcttgcgcccattgtgcaatattccccactgctgccccccccgaaggagtcaac
aatattgcacatggcaaccttccccccggtttttaaaaaaaaaattttttttttttaatttttttgggggggggggggttgttatttttttttttttttttcaaaa
aaaaaactctcaatccaaaaaaaaaattaaaaaa

K-Hf-H2 (*Lysobacter capsici*) nucleotide sequence from primers 27F/1492R

NNNNNNNNNNNNNNNNgcgNNNgcNNNNNatgcaagtcgaacggcagcacagaggagcttgctccttgggtgg
cgagtggcggacgggtgaggaatacgtcggaatctgcctatttgtgggggataacgtagggaaacttacgctaataccgcatacgacctac
gggtgaaagcggaggaccttcgggcttcgcgcagatagatgagccgacgtcggattagctagttggcggggtaaaggcccaccaaggc
gacgatccgtagctggtctgagaggatgatcagccacactggaactgagacacggtccagactcctacgggaggcagcagtggggaata
ttggacaatgggcgcaagcctgatccagccatgccgcgtgtgtgaagaaggccttcgggttgtaaagcacttttgtccggaaagaaaagttc
ccggttaatacccggggatcatgacggtaccggaagaataagcaccggctaacttcgtgccagcagccgcggtaatacgaagggtgcaa
gcgttactcggaattactgggcgtaaagcgtgcgtaggtggtttgttaagtctgatgtgaaagccctgggctcaacctgggaatggcattgga
aactggctgactagagtgcggtagagggtagtggaattcccggtgtagcagtgaaatgcgtagatatcgggaggaacatctgtggcgaag
gcgactacctggaccagcactgacactgaggcacgaaagcgtggggagcaaacaggattagataccctggtagtccacgccctaaacga
tgcgaactggatgttgggagcaacttggctctcagtatcgaagctaacgcgttaagttcgccgcctgggaagtacggtcgcaagactgaaa
ctcaaaggaattgacgggggcccgcacaagcggtggagtatgtggtttaattcgatgcaacgcgaagaaccttacctggccttgacatcca
cggaactttctagagatagattggtgccttcgggaaccgtgagacaggtgctgcatggctgtcgtcagctcgtgtcgtgagatgttgggttaa
gtcccgcaacgagcgcaacccttgtccttagttgccagcacgtaatggtgggaactctaaggagaccgccggtgacaaaccggaggaag
gtggggatgacgtcaagtcatcatggcccttacggccagggctacacacgtactacaatggtagggacagagggctgcaaacccgcgag
ggcaagccaatcccagaaaccctatctcagtccggatcggagtctgcaactcgactccgtgaagtcggaatcgctagtaatcgcagatcag
cattgctgcggtgaatacgttcccgggccttgtacacaccgcccgtcacaccatgggagtttgttgcaccagaagcaggtagcttaaccttcg
ggagggcgcNNNNNNNggtgtggccgNNNNNNNNNNNNNN